# Biological and ecological factors contributing to the successful use of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) for the control of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) under South African conditions

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## Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:

Date:

## Abstract

Codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) is a devastating pest of pome fruit in temperate regions of the world. Control of this pest, previously involved the extensive use of broad-spectrum insecticides. However, concerns over human safety, environmental impact, widespread dispersal of resistant populations of codling moth and the sustainability of synthetic pesticides in agroecosystems, has encouraged the development and use of alternative environmentally-friendly pest management technologies including the use of entomopathogenic nematodes. These nematodes are lethal pathogens of insects and belong to the families Steinernematidae and Heterorhabditidae, and are ideal candidates for incorporation into the integrated pest management programme currently being developed for residue-free pome fruit production in South Africa. The biological and ecological factors pertaining to the successful use of these nematodes for the control of codling moth were investigated in this study. Their use for bin-disinfestations was evaluated, focusing on the optimum handling conditions to ensure the survival and subsequent efficacy of the nematodes. The study proved that the local isolate SF41 of *Heterorhabditis zealandica* Poinar 1990 could be used for successful bin-disinfestation. The use of the same nematode isolate was also investigated for the disinfestation of mulch layers of diapausing codling moth larvae. An insect containment device which allowed for direct trial efficacy evaluation was identified and ecological factors pertaining to the successful use of nematodes for mulch disinfestation were investigated. The biological control potential of local nematode isolates, which had previously never been tested against codling moth larvae, was investigated in the laboratory under conditions as can be expected during orchard applications. The efficacy of the selected isolates was confirmed in field experiments. Innovative insect containment methods for above-ground trial efficacy evaluation in the field were investigated. Desiccation proved to be the most limiting factor to the survival and subsequent efficacy of the nematodes during field applications in temperate regions. The effect of low moisture levels on *H. zealandica*'s efficacy to control diapausing codling moth larvae was subsequently investigated and a starch-based formulation was further tested to overcome the issue of desiccation. Conclusive results indicated that there were several biological and ecological factors influencing the survival of nematodes and illustrated how these factors could be manipulated to overcome these issues and thereby ensure the efficacy of treatments. This is the first report of its kind to comprehensively investigate the use of South African entomopathogenic nematodes for the control of diapausing

codling moth larvae and all results emanating from the study can be integrated into a framework for the commercial use of these nematodes in this regard in future.

## Opsomming

Kodlingmot, *Cydia pomonella* (L.) is ‘n ernstige sleutelplaag in appel- en peerboorde in gematigde klimaats gebiede wêreldwyd. In die verlede is hoofsaaklik breëspektrum insektedoders gebruik vir die beheer van hierdie plaaginsek. Maar, kommer oor veiligheid vir die mens, impak op die omgewing, verspreiding van weerstandbiedende populasies van kodlingmot en beperkte volhoubaarheid van sintetiese plaagdoders het die ontwikkeling en gebruik van alternatiewe plaagbeheer tegnologieë, insluitend die gebruik van entomopatogeniese nematodes, genoodsaak. Entomopatogeniese nematodese horende tot die families Steinernematidae en Heterorhabditidae, is ideale kandidate vir insluiting in die geïntegreerde plaagbestuur programme wat huidiglik ontwikkel word vir gebruik in plaaslike boorde met die uiteindelike doel om residu-vrye vrugte te produseer. In hierdie studie word die biologiese en ekologiese faktore bestudeer wat die sukses van ‘n nematode-toediening gemik op kodlingmot beïnvloed. Hierdie studie het bewys dat die lokale SF41 isolaat van *Heterorhabditis zealandica* Poinar 1990 gebruik kan word om vrugtekratte te disinfesteer van kodlingmot. Die gebruik van dieselfde isolaat vir die disinfestasie van deklae is ook ondersoek. ‘n Metode van insek-inkamping is ook ontwikkel wat die evaluering van toedienings vergemaklik en meer effektief maak. Die omgewings-toestande wat ook bydrae tot die oorlewing en gevolglike sukses van ‘n toediening is ook ondersoek. Die biologiese beheer potensiaal van ‘n paar lokale isolate wat nog nooit voorheen teen kodlingmot getoets is nie, is ook bestudeer. Die isolate se effektiwiteit is ook bevestig in veldproewe en insek-bekampings metodes wat meer van toepassing is vir bogrondse plaaginsekte is ook geïdentifiseer. Resultate dui daarop dat vogverlies en gevolglike uitdroging van nematodes die grootste beperkende faktor is vir hierdie tipe toedienings in gematigde gebiede en ‘n stysel-gebaseerde formulasie is dus ondersoek om hierdie probleem te oorkom. Die uiteindelike gevolgtrekking van die studie was, dat alhoewel daar verskeie biologiese en ekologiese faktore is wat die oorlewing van nematodes beperk, daar tog verskeie maniere is om hierdie faktore te manipuleer en sodoende te oorkom, wat bydrae tot die uiteindelike sukses van ‘n toediening. Hierdie is die eerste studie wat werklik die praktiese gebruik van lokale entomopatogeniese nematodes vir die beheer van kodlingmot ondersoek en alle bevindinge kan geïntegreer word in toekomende riglyne vir die kommeriële gebruik van nematodes vir die beheer van kodlingmot.

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***"They said it would be worth it, they never said it would be easy"***

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# CHAPTER 1

# General Introduction

To place the study of using entomopathogenic nematodes for the control of codling moth in context, it was necessary to review three distinct, but pertinent, topics. Firstly, aspects of codling moth origin and dispersal, host range, pest status, biology, damage, monitoring and control must be known. Secondly, entomopathogenic nematode identification, host range, distribution, registration and biology must be reviewed and thirdly, the use of entomopathogenic nematodes for the control of codling moth, including those factors that influence their successful application, require addressing in order finally to formulate the main aims for the study.

## The codling moth

The moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Figure 1) was given the vernacular name of 'codling moth' by Wilkes in 1747, referring to codlings, elongated, greenish English cooking apples. The first definitive account of the species in the Netherlands was noted in 1635 by Jean Goedaerdt, who provided illustrations of the larva and moth. He referred to the codling moth as the 'pear eater' (Barnes 1991). From then on, until four centuries later, this insect has been a key pest of pome fruits in orchards worldwide.

**Figure 1.** Codling moth adult, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae).

It is speculated that apples and pears originated from Central Asia and a section of Europe, and, as the codling moth is closely associated with the fruit, it's origin, therefore appears to be Eurasian. The exact centre of dispersion has, however, been hidden by the passage of time (Barnes 1991). Along with the cultivation of said crops, codling moth has generally spread around the world, principally during the 18th and 19th centuries. A map illustrating the general distribution of codling moth is presented in Figure 2 (CABI 2011).

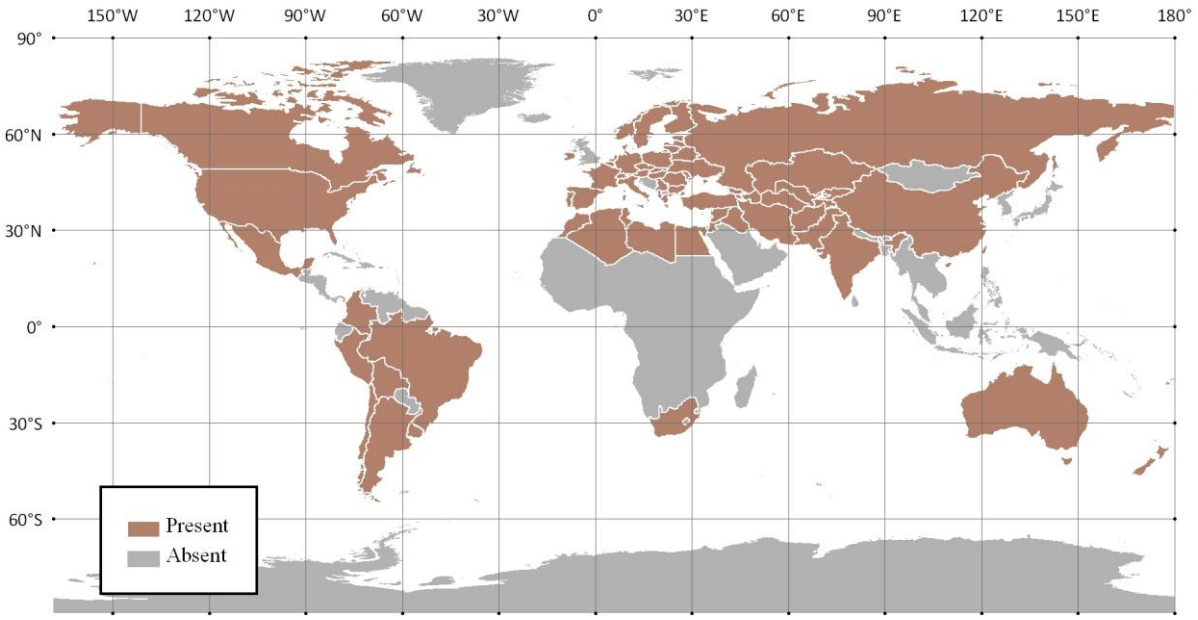


**Figure 2**. General distribution of the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). Shading of a country indicates that the organism has been recorded as being present in at least some part of that country.

Codling moth was first reported from Graaff-Reinet in South Africa around 1885, when it supposedly arrived in apples carried by a tourist stopping over at Madeira on his way to the Cape (Lounsbury 1898). Several initial urgent attempts were made to prevent the pest from spreading throughout the country (Lounsbury 1897). However, the pest readily established itself in fruit production areas surrounding Graaff-Reinet, including Nieu-Bethesda and thereafter throughout the Western Cape (Lounsbury 1899). Attempts and regulations to prevent the further spread of codling moth were eventually abandoned around 1918 (Lounsbury 1918), and, since then, codling moth has become an established key pest in pome fruit orchards throughout South Africa (Giliomee and Riedl 1998).

The codling moth is primarily associated with apples, as it is not only one of its original hosts, but also the one that is most susceptible to attack. Pears, quinces, walnuts, apricots, plums, peaches and nectarines are also readily attacked by this pest (Riedl 1983; Barnes 1991; CABI 2011). In 1983, codling moth was ranked as the fifth most important plant-feeding pest species in South Africa (Moran 1983), and today it is still regarded as a key pest of major concern in most pome fruit orchards throughout South Africa.

Codling moth passes through four developmental stages (Figure 3). Adult female moths (wingspan ± 2 cm) generally deposit their eggs (± 2 mm in diameter) singly on the fruit or on the foliage near the fruit, and occasionally also on the wood of trees. Eggs hatch after three to seven days, and neonate larvae (± 1 cm) wander in search of fruit to attack, occasionally feeding on foliage if the search is prolonged (Blomefield 2003). They penetrate the skin of the fruit and initially feed near the fruit surface for approximately two days, before moving towards the core of the fruit as second-instar larvae. There they feed on the developing seeds. They pass through five larval instars inside the fruit, emerging after 18 - 40 days to find a hidden and dry site in which to spin their cocoons (Welter 2008). These sites include dry areas under loose bark on trees, in pruning wounds, in litter at the base of trees, or in nearby woodpiles and fruit bins (Blomefield 2003; Cossentine *et al.* 2004). In early summer, cocooned larvae develop through pupae to maturity in roughly two weeks. As the days shorten in late summer, larvae enter a diapause phase and pass the winter months as mature larvae and prepupae in cocoons. Temperature is considered to be the most significant factor influencing the phenology of codling moth (Audemard 1991). The rate of development is dependent on accumulated degree-days above a base threshold of development, which for codling moth is 10°C (Riedl 1983), and

which is comprehensively described by Blomefield (2003) for both the embryonic and immature stages of codling moth under local conditions. Heat units accumulated for phenology models are accumulated from a 'biofix' in spring, which is the first consistent catch of codling moth males in pheromone traps. Codling moth generally has between one and four generations per year, depending on climate (Barnes 1991; Welter 2008). In South Africa, up to four generations of codling moth can occur per growing season (Pringle *et al.* 2003), with larval feeding activity extending from August through to April (Myburg 1980).

**Figure 3.** Developmental stages of the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae).

Crop losses caused by codling moth on pome fruit are difficult to assess, as the methods used are inadequate and not directly comparable (CABI 2011). Vickers and Rothschild (1991) report that codling moth damage in commercial orchards can be kept below 2% with the correct use of broad-spectrum insecticides. However, if left untreated, the infestation potential of codling moth can dramatically increase, especially in temperate regions, where two or more generations can occur (Geier 1964). The infestation potential of codling moth in South Africa has been reported to be one of the highest in the world, where up to 80% infestation of fruit in orchards has been recorded (Myburg 1980). Damage ranges from shallow feeding wounds, causing scarring of the fruit, to direct feeding damage to the pulp or seeds, or from indirect contamination of the fruit by larval faeces accumulating around the entrance point (Welter 2008). Current local export fruit production quality standards do not tolerate codling moth damage on apples and pears (www.nda.co.za).

**Figure 4.** Visual damage (feeding scars and larval frass) caused by the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) to an apple.

An effective monitoring programme forms an essential part of the control strategy to manage codling moth in commercial orchards in the current growing season, while also helping to predict the expected degree of infestation in the subsequent growing season (Riedl *et al.* 1998). General detection and inspection methods include visual assessments of leaves for codling moth eggs, fruit damage assessments during summer, the cardboard banding of trees during autumn and winter to estimate larval population numbers and the use of pheromone traps to establish adult population numbers (CABI 2011). In South Africa, fruit damage assessments by means of scouting and pheromone trapping are the most widely used monitoring methods (Pringle *et al.* 2003).

Previous codling moth control measures were predominantly based on the use of broad spectrum insecticides, particularly organophosphates (Riedl *et al.* 1998). Concerns over personal safety, environmental impact, the widespread dispersal of resistant populations of codling moth and the sustainability of synthetic pesticides have encouraged the development and use of alternative pest management technologies within an integrated pest management strategy (Blomefield 2003; CABI 2011). Cultural control practices include removal of - or alternatively spatial separation from - secondary sources of infestation, such as wooden-bin stacks and non-commercial host plants. Biological control options include use of codling moth granulosis virus and insect growth regulators. Host plant resistance, pheromone-based mating disruption, and sterile insect technique have also been noted to be effective codling moth control methods in certain areas (CABI 2011), as has, recently, the use of entomopathogenic nematodes (Lacey and Unruh 1998). In South Africa, all of the

aforementioned control practices are implemented as part of an area-wide codling moth control programme in commercial pome fruit orchards, with the exception of the use of entomopathogenic nematodes. The latter is currently still in its developmental research phase (Riedl *et al.* 1998; Giliomee and Riedl 1998; Addison 2005; De Waal 2008). Insecticides, unfortunately, remain the primary means of controlling codling moth, with up to 30 different registered insecticides currently being used for control in South Africa (www.hortgro.co.za).

## Entomopathogenic Nematodes

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae are lethal pathogens of insects. These obligate pathogens contribute to the regulation of natural populations of insects in the soil, but the main interest that has been expressed in them regard their use as inundatively applied microbial agents for augmentative biological control.

Several technologies (including both morphological and molecular methods) are available for the accurate identification of entomopathogenic nematode taxa (Nguyen and Hunt 2007). Morphological characteristics can be used to distinguish several species of *Steinernema,* but for *Heterorhabditis* a lack of differentiating morphological features makes this approach too difficult, resulting in molecular characterization being imperative (Hunt 2007). Attributing to the necessity for using molecular methods for the identification of entomopathogenic nematode species, is the significant amount of environmental and host-induced morphological variation displayed by the nematodes (Nguyen and Smart 1996; Hominick *et al.* 1997).

Under optimal laboratory conditions, most entomopathogenic nematode species readily infect a broad range of insects. In the field, however, these nematodes attack a significantly narrower host range as conditions are not always optimal, host contact is not always assured and environmental or behavioural barriers to infection may exist (Kaya and Gaugler 1993; Adams *et al.* 2007). These nematodes are adapted to the soil environment, and thus their principle hosts are the soil stages of insects. They can, however, also be used for the treatment of insects that occur above-ground

(Koppenhöfer 2007). No significant acute or chronic toxicity to humans or other vertebrates has ever been reported, and no noteworthy long-term impact on non-target invertebrate populations has been established. So far, researchers have been unable to identify any safety considerations that should prevent the use of entomopathogenic nematodes as biological control agents (Akhurst and Smith 2002; Ehlers 2005).

Entomopathogenic nematodes are widespread and have been recovered from soils throughout the world (Kaya 1990). Numerous surveys have documented their dispersal in both cultivated and uncultivated soils (Hominick *et al.* 1996; Hominick 2002). The only continent where they have not been found is Antarctica (Griffin *et al.* 1990).

In South Africa, the first occurrence of a *Steinernema* species was documented in 1953, retrieved from the maize beetle *Heteronychus arator* (Fabricius) (=*H. sanctae-helenae* Blanch.) in a maize field in Grahamstown, Eastern Cape Province (Harrington 1953). Several years later, in a survey that was conducted to obtain effective nematodes for the possible control of the African sugarcane stalk-borer, *Eldana saccharina* Walker, many isolates of both *Heterorhabditis* and *Steinernema* were found, but not identified to species level (Spaull 1988; 1990; 1991). The first identification of an entomopathogenic nematode to species level was that of *H. bacteriophora* Poinar 1975 from the Western Cape Province in 1996 (Grenier *et al.* 1996). In 2003 a survey was conducted, documenting the occurrence of entomopathogenic nematodes in the south-western parts of South Africa. Several isolates of *Heterorhabditis* were found and only one species of *Steinernema* (Malan *et al.* 2006). The latter species of *Steinernema* was subsequently described as *S. khoisanae* Nguyen, Malan and Gozel 2006 (Nguyen *et al.* 2006). Another new species obtained from this survey was a *Heterorhabditis* species, subsequently described as *H. safricana* Malan, Khuong, De Waal and Tiedt 2008 (Malan *et al.* 2008). Hatting *et al.* (2009) conducted a survey from different habitats in several geographic regions of South Africa from 2003 to 2005 and recovered four *Steinernema* spp., including *S. khoisanae,* three new undescribed species, and only one *Heterorhabditis* sp., *H. bacteriophora*. In 2008, De Waal (2008) obtained several isolates of *Heterorhabditis* and *Steinernema* from predominantly Western Cape soils in an attempt to find a suitable nematode for the control of codling moth, including two new *Steinernema* spp., which have not yet been described. The most recent survey was conducted by Malan *et al.* (2011) in citrus orchards throughout South Africa, whereby numerous isolates of both *Heterorhabditis* and *Steinernema* were isolated, of which the most

noteworthy was *S. yirgalemense* Nguyen, Tesfamarian, Gozel, Gaugler and Adams 2005, a new species of *Steinernema*, recently described as *S. citrae* (Stokwe *et al.* 2011) and an unknown *Heterorhabditis* sp. To date, the following species have been reported from South African soils: *H. bacteriophora* (Grenier *et al.* 1996), *H. zealandica* (Malan *et al.* 2006), *H. safricana* (Malan *et al.* 2006; Malan *et al.* 2008), *S. citrae* (Stokwe *et al.* 2011; Malan *et al.* 2011), *S. khoisanae* (Nguyen *et al.* 2006; Malan *et al.* 2006) and *S.yirgalemense* (Malan *et al.* 2011).

Entomopathogenic nematodes require registration in South Africa, unlike in several other countries (Ehlers 2005). The current tabled proposed registration guidelines, which have not yet been legislated, suggest that entomopathogenic nematodes will, however, be exempt from certain data requirements, including efficacy trials. Registration should, therefore, not be onerous. Whether the strain is a local isolate, or whether it is imported, will make no difference to the registration requirements, as such requirements will be the same for both. Importation-requirements have already been legislated as part of an amendment to Act 18 of 1989. Under the Agricultural Pests Act (Act 36 of 1947), it is clearly stated that importation of a species of organism is prohibited if exotic. It is, therefore, only permissible, with governmental approval, to import species that have previously been reported from local soils.

Steinernematids and heterorhabditids have a non-feeding, free-living, developmentally-arrested third-stage infective juvenile (IJ), or dauer juvenile, that infects the insect host in the natural soil environment. The IJ is sheathed in a second-stage cuticle that is easily lost in steinernematids, but which is retained for longer periods in heterorhabditids. Both *Heterorhabditis* and *Steinernema* are mutualistically associated with bacteria of the genera *Xenorhabdus* and *Photorhabdus,* respectively (Boemare 2002). Upon infecting the insect host through natural openings (mouth, anus, spiracles) or thin areas of the host's cuticle (common only in the heterorhabditids that gain entry by abrading the intersegmental membranes of the insect using a dorsal tooth), they penetrate into the host's haemocoel and release the bacterium from their intestine. The bacterium then propagates and produces substances that rapidly kill the host (normally within 24 - 48 h) through septicemia (Poinar 1990; Dowds and Peters 2002). The nematode feeds on the bacterial cells and host tissue that have been metabolised by the bacterium and, depending on host size, completes 1 - 3 generations. As food resources in the host cadaver are depleted, a new generation of IJs is produced and emerges from the host cadaver into the soil in search of a new host. Steinernematids and heterorhabditids

differ in their mode of reproduction. Heterorhabditids are hermaphroditic in the first generation (± 1 mm) (Figure 5) and amphitic in the following generations, as opposed to every generation of all but one steinernematid species that reproduce by amphimixis (Hazir *et al.* 2003; Griffin *et al.* 2005). Entomopathogenic nematode species employ different foraging strategies to locate and infect hosts. Ambushing nematodes nictate during foraging by raising nearly all of their body off the substratum by standing on their tails and latching onto passing insects, as opposed to cruising nematodes that orientate themselves to volatile host cues by moving through the soil towards the host. Most nematode species adopt an intermediate foraging behaviour and are, therefore, known as intermediate strategists (Griffin *et al.* 2005).

**Figure 5.** First-generation hermaphroditic nematodes visible from a dissected final-instar diapausing codling moth larva.

## Obstacles to the use of entomopathogenic nematodes for codling moth control

Although entomopathogenic nematodes are generally pathogenic to a wide variety of insects, successful commercialisation and application on a commercial basis has been limited to relatively few target insects (Grewal and Georgis 1999; Shapiro-Ilan *et al.* 2002).

Codling moth has not only proved to be susceptible to entomopathogenic nematodes in research experiments (Kaya *et al.* 1984; Lacey and Unruh 1998; Lacey and Chauvin 1999; Vega *et al.* 2000; Unruh and Lacey 2001; Cossentine *et al.* 2002; Lacey and Shapiro-Ilan 2003; Lacey and Unruh 2005; Lacey *et al.* 2005; Lacey *et al.* 2006a; Lacey *et al.* 2006b; Lacey *et al.* 2007; De Waal 2008; Lacey *et al.* 2010), but is also one of the few examples of entomopathogenic nematodes being used on a commercial basis to help control this insect pest in certain parts of the world.

Literature indicates that *S. feltiae* and *S. carpocapsae* are the most promising, and thus the most widely used, nematode species for the control of codling moth (Kaya *et al.* 1984; Lacey and Unruh 1998; Vega *et al.* 2000; Unruh and Lacey 2001; Cossentine *et al.* 2002; Lacey and Unruh 2005; Navaneethan *et al.* 2010). This is partially due to their commercial availability in some countries, but also because of their biological characteristics (Lacey and Unruh 1998; Vega *et al.* 2000). In South Africa, however, neither of these two species has been isolated to date, and as was previously mentioned, current legislation prohibits the import of exotic species into the country. Consequently, all previous local research has been conducted using local nematode isolates.

The most sensible stage for controlling the codling moth with nematodes is that of the cocooned diapausing larvae, which occurs in late autumn, winter and early spring in temperate areas. During this period, the entire codling moth population overwinters under loose bark and in pruning wounds on trees, in litter at the base of trees, and in nearby woodpiles and fruit bins, all of which are sites that are reasonably favourable to the survival of entomopathogenic nematodes (Lacey and Unruh 2005). The elimination, or significant reduction, of the codling moth population at this stage would provide complete or substantial protection to fruit early in the following growing season, emphasising the importance of either targeting the larvae in spring before they pupate, or in autumn once they have entered diapause (Lacey *et al.* 2005; Navaneethan *et al.* 2010).

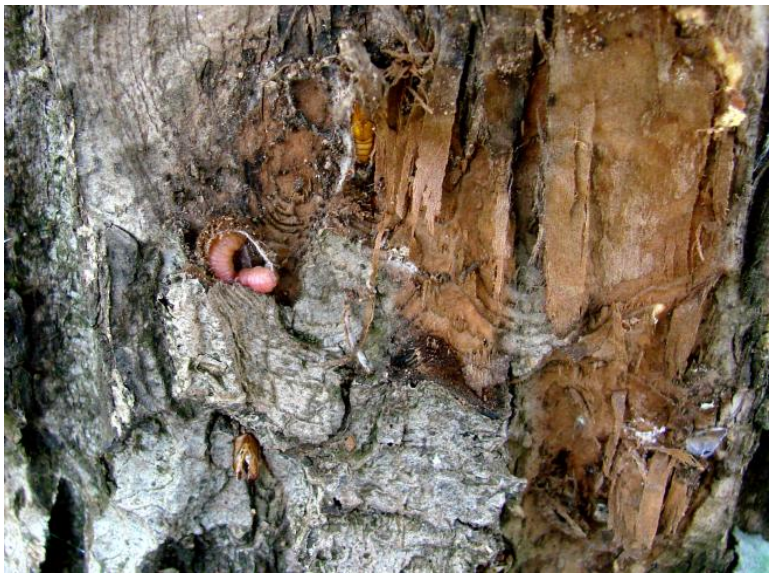

**Figure 6.** A diapausing codling moth larva and pupae occurring underneath loose pieces of bark on trees during late-spring.

The first use of entomopathogenic nematodes for the control of codling moth in South Africa was documented by Malan and Addison (2008) as part of a preliminary laboratory bioassay that was aimed at selecting a promising local isolate for future use. *Heterorhabditis zealandica* (SF41) was selected from this study, and was further screened against codling moth in laboratory bioassays, together with 21 other isolates obtained from a previous survey undertaken by De Waal (2008). Again, *H. zealandica* proved to be the most efficacious isolate, and was thereupon evaluated in laboratory bioassays and field experiments (De Waal 2008).

Results from the aforementioned study indicated the *H. zealandica* isolate to be relatively effective for the control of codling moth under local conditions. However, several impeding factors were highlighted, which need to be addressed in future research in order to ensure the enhanced efficacy of a treatment in local pome fruit orchards. Further opportunities for the control of codling moth with the nematodes were also identified, with these findings corroborating some of the scenarios and factors listed in several literature sources. The factors concerned, include both extrinsic factors, comprising factors in the orchard environment, and intrinsic factors, referring to the biological characteristics of the nematodes (Koppenhöfer 2007; De Waal 2008).

As previously mentioned, nematodes occur naturally in soil, where they are buffered from extreme environmental conditions. As the proposed codling moth stage to target with the use of nematodes is

the diapausing codling moth larvae, which occurs above-ground, the main obstacles to overcome would, therefore, be climatic conditions in the orchard environment, which could hamper the survival and subsequent performance of nematodes. These factors have previously been indicated as significantly hampering the efficacy of treatments (Lacey and Unruh 1998; Glazer 2002; Wright *et al.* 2005). Sources of re-infestation (such as bin stacks and litter at the base of trees) have also been noted to limit the efficacy of treatments (Lacey and Chauvin 1999; Lacey *et al.* 2006b).

Optimum moisture levels pertaining to both the relative humidity in the surrounding orchard environment (the macro-environment) and the cryptic habitats on trees, where larvae mainly tend to reside (the micro-environment), should favour nematode survival for an adequate period of time to ensure the efficacy of an application aimed at diapausing codling moth larvae (Navaneethan *et al.* 2010). From the time of application, nematodes have to enter the cryptic habitat where the diapausing larvae resides, to penetrate the surrounding cocoon, and to enter the host before the habitat dries out (Lacey *et al.* 2006a). Nematode strains can also be genetically improved to tolerate certain relatively low levels of moisture (Mukuka *et al.* 2010), however, manipulation of the habitat where the nematodes will be applied still seems to be the most practical solution to the existing problem (Webster 1973; Glazer 2002; Navaneethan *et al.* 2010; Lacey *et al.* 2010). Nematode-formulation through the addition of certain additives with water-holding properties has proved to prevent nematodes from rapid desiccation and thereby to increase the efficacy of treatments (Glazer 2002; Navaneethan *et al.* 2010; Lacey *et al.* 2010). Moisture levels in the orchard and on trees can also further be increased by incorporating existing orchard irrigation systems and standard hydraulic application equipment technology. It is also advisable not to make applications on windy days, as the blowing of wind shortens the drying time of the substrate onto which the nematodes are applied (Unruh and Lacey 2001). Previous laboratory experimentation using the local SF41 isolate *of H. zealandica*, showed that optimal performance was only recorded when maximum levels of humidity (> 95% RH) were maintained for at least 5 h in the macro-environment (De Waal 2008). However, no work has been conducted with any other local isolates to illustrate their performance under low moisture levels in the micro-environment.

Low temperatures during autumn, winter and spring have been noted to limit the efficacy of nematode treatments aimed at diapausing codling moth larvae (Glazer 2002; Lacey *et al.* 2006a). Survival temperatures for the nematodes generally range from 5°C to 15°C (Georgis 1990). Nematodes

become sluggish at low temperatures (<10 - 15°C) and are inactivated at higher temperatures (> 30 - 40°C), with optimal performance ranging between 20°C and 30°C (Koppenhöfer 2007), depending on the duration of exposure. Proposed solutions for overcoming the detrimental effect of lower temperatures include the temperature tolerance improvement of strains, using genetic selection (Ehlers *et al.* 2005) and alternatively or additionally, using such species as *S. feltiae* that have been proved to be efficacious at temperatures below 15°C (Grewal *et al.* 1996).

In temperate regions such as South Africa, daily autumn, winter and spring temperatures can range within the optimal temperature range (20 - 30°C), which is conductive to nematode activity. However, during the evening, temperatures generally drop below 20°C, which would hamper the efficacy of treatments. *Heterorhabditis zealandica* is the only local isolate that has been evaluated for use against codling moth at low temperatures in both laboratory and field experiments (De Waal 2008). However, temperatures tested in the laboratory remained at constant levels, and the field experiment was not conducted during the proposed seasonal time of application. Therefore, further investigation is warranted, as well as an altered experimental design, including variable temperatures, in order to reach sound conclusions.

In addition to diapausing on trees in cryptic habitats, as was discussed above, codling moth larvae also tend to diapause in litter at the base of trees, especially in apple orchards, where trees have a smooth bark, thus offering less cryptic habitats in which larvae can spin their cocoons. Mulches would, therefore, be an ideal overwintering site and, if not treated, could act as a source of codling moth infestation. As mulching is becoming increasing popular in South African orchards, it would be beneficial to investigate the use of nematodes for the control of diapausing codling moth larvae in mulches, as proposed by Lacey *et al*. (2006b).

Wooden fruit bins used during harvesting and to transport and store fruit have also been reported to be infested with diapausing codling moth larvae (Higbee *et al.* 2001). The bins can act as an external source of codling moth infestation if left untreated (Cadré and Minks 1995; Higbee *et al.* 2001; Lacey *et al.* 2005), which would hamper the efficacy of density-dependent codling moth control practices, such as mating disruption and sterile insect release. Most of the bins used in the South African fruit industry are wooden and have been reported to be infested with codling moth. If the bins could be treated with nematodes, as has been proposed in several studies (Lacey and Chauvin 1999;

Cossentine *et al.* 2002; Lacey *et al.* 2005), it would be beneficial to the areawide and integrated approach to codling moth control currently implemented throughout fruit-producing areas in South Africa, including to the aforementioned density-dependent control strategies.

**Figure 7.** Wooden fruit bins infested with codling moth acting as external sources of infestation for orchards in close proximity.

Although all entomopathogenic nematodes belong to the same group, they do not possess the same characteristics, and therefore do not hold the same potential as do biological control agents (Morton and Garcia-del-Pino 2009). Species characterisation is, therefore, an important action to be taken during the selection of an appropriate isolate for the control of a target insect in order to match the biological and ecological characteristics of the nematode isolate to the conditions associated with the target insect (Koppenhöfer and Kaya 1999; Shapiro-Ilan *et al.* 2002; 2009; Morton and Garcia-del-Pino 2009).

In the case of codling moth, the most suitable nematode isolate would not only have to be virulent against codling moth, but would also have to be effective at relatively low temperatures and moisture levels, as can be expected in an orchard during application, when targeting the suggested diapausing larval stage. The isolate would also have to possess an active searching behaviour in order to locate larvae residing in cryptic habitats on trees. It is imperative that selected local isolates also be

characterised in terms of the above-mentioned criteria to ensure the eventual successful application of such for the control of codling moth in local orchards.

## Aims of the study

In view of the above-mentioned findings that have been covered in the literature reviewed, the general aim of the current study was to evaluate local entomopathogenic nematode isolates under South African conditions in terms of the environmental and biological factors contributing to the success or failure of an application.

The specific objectives of the study were, therefore, to evaluate:

1. The key elements involved in the successful disinfestation of wooden fruit bins from diapausing codling moth larvae, using nematodes.
2. The use of nematodes in conjunction with different mulches for the improved control of diapausing codling moth larvae.
3. The required characteristics for nematodes to be effective biological control agents of diapausing codling moth larvae.
4. The use of a nematode formulation for improved performance of nematodes to control diapausing codling moth larvae.

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# CHAPTER 2

## Key elements in the successful control of diapausing codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae) in wooden fruit bins with a South African isolate of *Heterorhabditis zealandica* (Rhabditida: Heterorhabditidae)*

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### Abstract

The non-insecticidal control strategies currently being implemented in South African orchards for the control of codling moth, *Cydia pomonella* (L.) may be hampered by wooden fruit bins being infested with diapausing codling moth larvae, acting as a potential source of re-infestation. Key factors contributing to the success or failure of an entomopathogenic nematode application were investigated using the SF41 isolate of *Heterorhabditis zealandica* in laboratory bioassays with wooden minibins. Under operational conditions, an application rate of 100 IJs/ml ($LD_{90}$ = 102 IJs/ml) effectively controlled codling moth larvae in these bins, and for further laboratory bioassays, the $LD_{50}$ value of 18 IJs/ml (≈ 25 IJs/ml) was identified as the discriminating dosage. Maximum mortality was attained when bins were pre-wet for at least one minute (> 90% RH) and maintained at maximum humidity (> 95% RH) post-treatment for at least three days ($LT_{90}$ = 73 h), to ensure nematode survival and subsequent satisfactory infection of diapausing codling moth larvae. Tarping bins achieved the desired high level of humidity required. Furthermore, adjuvants (specifically Reverseal 10™) also improved an application. The study conclusively illustrated that if all the above-mentioned conditions are met, *H. zealandica* has the potential to successfully disinfest wooden fruit bins of codling moth.

## Introduction

Codling moth, *Cydia pomonella* (L.), is one of the most serious and widely distributed pest world wide on apples and pears (Barnes 1991). It has been a key pest in South African pome fruit orchards since it was first reported there in 1885 (Lounsbury 1898; Giliomee and Riedl 1998). Codling moth damage results in unmarketable produce. Codling moth has up to four generations per growing season in South Africa (Pringle *et al.* 2003), making the fruit infestation potential of this pest one of the highest in the world (Myburgh 1980).

When final-instar larvae emerge from fruit in autumn, they search for a suitable overwintering site in which to spin their cocoons. Once they have selected a site, they spin a cocoon which provides them with protection throughout the winter until spring when the larvae pupate and develop into adult moths. These preferred overwintering sites are normally dry and sheltered, such as under loose pieces of bark on trees, in litter at the base of trees, or in nearby woodpiles and wooden fruit bins (Blomefield 2003; Cossentine *et al.* 2004; Higbee *et al.* 2001).

Control of this pest was previously predominantly based on the use of broad spectrum insecticides (Riedl *et al.* 1998). An integrated approach to codling moth control is currently being used in commercial orchards in South Africa. Some of the tactics integrated into this strategy, such as mating disruption and the sterile insect technique (Pringle *et al.* 2003; Addison 2005), are density-dependent (Cardé and Minks 1995; Judd and Gardiner 2005). Wooden fruit bins used during harvesting and to transport and store fruit have been reported to be infested with diapausing codling moth larvae (Higbee *et al.* 2001). The control of codling moth, using the above-mentioned density-dependent and non-insecticidal practices, can therefore be compromised due to the invasion of orchards by moths emerging from wooden fruit bins which were previously infested and placed in or near orchards prior to harvesting (Cardé and Minks, 1995; Higbee *et al.* 2001; Lacey *et al.* 2005). Bin treatments will thus contribute to lowering the pest population levels and therefore indirectly benefit mating disruption and the sterile insect release programme (Lacey *et al.* 2005).

Several methods have been investigated for disinfesting wooden fruit bins of diapausing codling moth larvae, including: timing of bin placements in orchards as a preventative approach, using plastic bins instead of wooden bins, fumigating bins with methyl bromide (Dentener *et al.* 1998), the use of the biofumigant fungus *Muscodor albus* Worapong, Strobel and Hess (Ascomycota: Xylariales) as an alternative to broad spectrum chemical fumigants (Lacey *et al.* 2009), fumigating bins with carbon dioxide (Cossentine *et al.* 2004), hot water dips (Hansen *et al.* 2006) and heat treatments (Higbee *et al.* 2001).

The use of entomopathogenic nematodes (EPNs) for the disinfestation of wooden fruit bins from diapausing codling moth larvae is relatively well documented. Lacey and Chauvin (1999) demonstrated that final-instar codling moth larvae positioned in miniature wooden fruit bins can be an effective method of assessing nematode efficacy without the cumbersome size of normal commercial bins. Cossentine *et al.* (2002) investigated treatment of bins with *Steinernema carpocapsae* Weiser infective juveniles (IJs) by submersion or drenching, respectively. Lacey *et al.* (2005) investigated drenching bins with *S. carpocapsae* in an industrial bin washer. The use of EPNs to successfully control oriental fruit moth, *Grapholita molesta* (Busck), in miniature wooden fruit bins has also been documented by Riga *et al.* (2006).

Most of the research thus far conducted on EPNs for codling moth control has been with *S. carpocapsae* and *S. feltiae* Wouts, Mráček, Gerdin & Bedding (Lacey *et al.*, 2005). Neither of these species has been reported from local soils and current legislation in South Africa makes it difficult to obtain exotic isolates. Subsequently, under local conditions, it is more practical to work with local isolates, such as the SF41 isolate of *H. zealandica* Poinar. Several endemic EPN isolates have been screened in laboratory bioassays for the control of codling moth, and from these screenings SF41 *H. zealandica* was the most promising isolate for the control of codling moth, not only in the field, but also for treating infested wooden fruit bins (De Waal *et al.* 2009; Malan and Addison 2008). This isolate was therefore used in the current study.

The goal of this study was to expand on above-mentioned research and identify baseline requirements needed to successfully disinfest wooden fruit bins using *H. zealandica* under local operational conditions. The number of nematodes required to obtain satisfactory levels of codling moth control in

wooden fruit bins was determined by means of a concentration trial. Humidity is known to be one of the predominant factors affecting nematode desiccation and factors influencing humidity, including the pre-wetting period of bins, incubation humidity, the time of incubation and post-treatment bin tarping were investigated. The impact of three adjuvants on nematode efficacy was also evaluated.

## Materials and methods

### *Source of nematodes*

All experiments were conducted using the SF41 isolate of *H. zealandica*, originally isolated from a soil sample collected near Patensie, South Africa (Malan *et al.* 2006). Infective juveniles were produced in *Galleria mellonella* (L.) and/or *Tenebrio molitor* (L.) larvae at room temperature. Harvested nematodes were stored in 150 ml of filtered water in vented 500 ml culture flasks which were placed horizontally at 14°C and shaken weekly for aeration. Infective juvenile concentrations for all trials were quantified in the laboratory in filtered water, using procedures described by Kaya and Stock (1997), one hour before commencing each experiment.

### *Source of codling moth larvae and use as sentinels*

Codling moth diet and eggs were obtained from the Deciduous Fruit Producers Trust's Sterile Insect Release Codling Moth Rearing Facility in Stellenbosch, South Africa. From these eggs, larvae were reared on an artificial diet under diapausing conditions [photoperiod 10:14 (L:D), 25°C, 60% RH]. Fifth instar diapausing codling moth larvae were used for experimentation as this is the life stage that will eventually be targeted in wooden fruit bins and to avoid pupation during the test period.

### *Bioassay protocol*

Nematodes were evaluated in scaled-down wooden fruit bins (≈ 1/10 of the full-sized commercial wooden fruit bins), constructed from the same wood (2.2 cm diameter) used for commercial wooden fruit bins (Image 1).

**Image 1.** Scaled-down wooden fruit bins (18 x 25 x 35 cm) used for laboratory bioassays.

In each corner of the bin, a wooden plank (Image 2), also constructed from fruit bin wood, (17.6 x 6.5 cm) was affixed to the side and a supporting corner cove (9 x 9 x 14 cm) was screwed on top of each of the planks in each corner (Image 3). Each plank was sawn in three parts and bolted together to facilitate easy removal of codling moth larvae from the planks at the end of each experiment. Twenty holes (5 mm diameter, 2 cm apart) were drilled into the wood along the groove, where the two pieces of wood met.

**Image 2.** Wooden planks (17.6 x 6.5 cm) used for laboratory experimentation.

**Image 3.** Wooden planks were place behind each corner support for each treatment bin.

Each plank was placed in a 2 L plastic container and twenty diapausing codling moth larvae were added to each of these, the lids were then closed and the larvae allowed to spin cocoons in the holes over a 24 h period (Malan *et al.*, 2009). Each bin consisted of four planks and each of these planks (containing 20 diapausing codling moth larvae), was considered as a sample. Minibins were pre-wet by immersing the entire bin in a cuboidal tank (Plastics for Africa, 36 cm wide, 38 cm long 29 x cm deep), filled with 36 L of water, for the desired period of time, depending on the experiment. Bins were then placed on a drying-rack to drip-dry. A second tank was used for the nematode dips. Nematodes were suspended in water at the desired concentration. Bins were then immersed in the tank containing nematodes under the required conditions. To prevent nematodes from settling, the suspension was mechanically stirred every two minutes after each bin was dipped using a wooden spoon. All experiments were conducted at room temperature in the laboratory. At the end of the treatment, bins were placed in black plastic bags lined with moistened paper towels to maintain a high relative humidity (95% RH) at 25°C throughout the test period. After incubation, the wooden planks were removed from the mini-bins, dismantled and codling moth larvae assessed for mortality by dissecting the larvae in Ringer's Solution to confirm nematode infection. Bins and planks were rinsed thoroughly and dried in a 60°C incubator after each experiment.

***Effect of infective juvenile concentration***

The effect of IJ concentration on the larvicidal concentration of *H. zealandica* was studied using 0, 6, 12, 25, 50 and 100 IJs/ml. Two bins (each containing four planks, with each plank housing 20 diapausing larvae) were treated for each of the test concentrations. Each bin was pre-wet for two minutes, drip-dried for another two minutes and then dipped into the specific test concentration solution. The experiment was repeated on two separate dates.

***Effect of pre-wetting period***

The effect of different pre-wet periods (0, 0.6, 1, 1.5 and 2.25 minutes) in water prior to the nematode treatment was investigated using the described bioassay system. After the initial pre-wet, planks were left to drip-dry for two-minutes and then dipped for one minute into the IJ suspension at a concentration of 25 IJs/ml. Two bins (each containing four planks, with each plank housing 20 diapausing larvae) were treated for each pre-wet period investigated. The experiment was repeated on two separate dates.

***Effect of humidity***

The effect of evaporation under four humidity regimes on the larvicidal activity of *H. zealandica* was studied using four planks (without the bins), each containing 20 diapausing larvae, for each humidity level being evaluated. Saturated suspensions in humidity chambers or closed environments were used to achieve 25% (potassium acetate), 50% (humidity in growth chamber), 75% (sodium chloride) and > 95% RH (closed plastic container lined with moistened tissue paper) (Winston and Bates 1960). Relative humidity was monitored throughout the trial period by placing a Hobo® H8 Pro Series data logger (Onset Computer Corporation, Massachusetts) into each humidity chamber. The planks were pre-wet for two minutes, drip-dried for another two minutes and then dipped into the IJ suspension for one minute at a concentration of 25 IJs/ml. Directly after treatment, planks were placed in the relevant humidity chamber or environment at 25°C in the dark and assessed after seven days. As control treatments for each humidity regime, an additional four planks (each containing 20 diapausing codling

moth larvae) were dipped in water only, prior to incubation. The experiment was repeated on two separate dates.

***Effect of incubation time***

To evaluate the effect of incubation time at > 95% RH, planks were used without the bins as described in the bioassay protocol. After treatment, all planks were incubated in closed plastic containers lined with moistened tissue paper (> 95% RH). Humidity was recorded using Hobo® H8 Pro Series data loggers in each plastic container. Three planks (each containing 20 diapausing codling moth larvae) were treated with nematodes for each incubation period investigated. For each incubation time, a fourth plank was dipped only in water to serve as a control treatment. The treatment planks were pre-wet for two minutes, drip-dried for two minutes and then dipped into the IJ suspension for one minute at a concentration of 25 IJs/ml. All planks were placed in the plastic containers after treatment and incubated at 25°C in the dark for 4, 6, 9, 13.5, 20.3, 30.4, 45.6, 68.3, 102.5 and 153 h. After each incubation period, the treated planks were removed from the containers, dismantled and the larvae removed. The larvae were rinsed with water under a tap to remove surface nematodes and placed in Petri dishes lined with moistened filter paper to allow nematode development for another five days. Mortality was assessed and thereafter dead larvae were dissected in Ringer's Solution to confirm infection. The experiment was repeated on two separate dates.

***Adjuvant Trial***

Before experimentation, adjuvants were first tested for any negative effects on nematodes. Approximately 200 IJs were suspended in 2 cm diameter watch glasses containing: Solitaire™ (polyether-polymethylsiloxane co-polymer / vegetable oil (EW) Safagric) at a concentration of 1 ml Solitaire™/L water or Reverseal 5/1™ (polymers 533 g/L, Farmkem) at a concentration of 0.5 ml Reverseal 5/1™/L water or Reverseal 10™ (mixed polymers 667 g/L, Farmkem) at a concentration of 1.2 ml Reverseal 10™/L water. Nematode survival was determined by noting the number of live nematodes at the start of the trial and again after 24 h. Nematodes not responding when prodded were noted as dead. The effect of the addition of an adjuvant on the larvicidal activity of *H. zealandica* for the treatment of bins was evaluated using the described bioassay. The adjuvants were added to

the nematode suspension at the above-mentioned concentrations. Bins were pre-wet for one minute, drip-dried for one minute and then dipped into an IJ suspension (containing the particular adjuvant being tested or in the case of the control: only water and nematodes) for one minute at a concentration of 20 IJs/ml. Two bins (each containing two planks, with each plank housing 20 diapausing larvae) were treated for each adjuvant or control treatment investigated, totaling four planks (each with 20 larvae per treatment). The experiment was repeated on two separate dates.

***Tarping Trial***

Bins were pre-wet for two minutes, drip-dried for two minutes and then dipped into an IJ suspension for one minute at a concentration of 25 IJs/ml. Bins were then stacked and a Hobo® H8 Pro Series data logger was placed inside each stack to record the relative humidity during the trial period. Twenty bins in total were used for this experiment. Each bin contained one plank with 20 diapausing codling moth larvae. Instead of placing the bins in black bags after the nematode treatment, half of the bins were stacked on top of each other and covered with a plastic tarpaulin sheet and the other half were left uncovered. All bins were kept in the laboratory at 25°C for the trial period. The experiment was repeated on two separate dates.

***Statistical analysis***

All statistical analyses were performed using the Statistica 8.0 software (Statsoft Inc., Tulsa OK, USA 2007). Data obtained from the pre-wetting period, humidity, adjuvant and tarping experiments were analyzed using a factorial ANOVA with trial test date and relevant treatments as separate factors. If there were no trial test date-treatment interactions, main effects (trial test date and treatment) were interpreted. Interaction effects were analyzed with Bootstrap multiple comparisons for comparing the interactions when residuals obtained in the ANOVA were not normally distributed (Efron and Tibshirani 1993). For the humidity trial, Abbott's correction factor (Abbott 1925) was used to correct for control mortality at each of the tested humidities and the resulting corrected mortality was used for the final ANOVA analysis. To statistically evaluate the concentration and incubation-period experiments, a Probit analysis was conducted using Polo PC (LeOra Software 1987). Test date data were pooled for the Probit analysis in both cases. Furthermore, for the Probit model, the number of control replications

for each treatment had no influence on the results of the analysis, because all the controls were lumped together, as were all the replications, for each treatment during the analysis. The $LD_{50, 90, 95, 99}$ values were estimated with their corresponding 95% fiducial limits. All values throughout the text were given with corresponding standard errors.

## Results

### *Effect of infective juvenile concentration*

All the concentrations tested caused some degree of larval mortality, except for the control (water only) treatment where no mortality was observed. The regression of probit mortality on the log of concentration for the pooled data fitted the model relatively well ($\chi^2$ = 7.82; df = 3; P = 0.05). The regression equation for the combined data was Y = 3.40 + 1.27[X], where Y = probit mortality and X = log (concentration). The $LD_{50}$, and $LD_{90}$ values in IJs/ml (and 95% fiducial limits) were 18 (10 – 28) and 187 (90 – 1052) IJs/ml respectively (Table 1).

**Table 1.** LD-values and their corresponding 95% fiducial limits obtained from a trial investigating the effect of different concentrations of *Heterorhabditis zealandica* (SF41) infective juveniles on codling moth larval mortality.

| LD* (% Mortality) | Value (IJs/ml) | 95 % Lower fiducial limit (IJs/ml) | 95 % Upper fiducial limit (IJs/ml) |
|---|---|---|---|
| 50 | 18 | 11 | 26 |
| 90 | 102 | 60 | 301 |
| 95 | 166 | 89 | 665 |
| 99 | 419 | 173 | 3015 |

* LD-values are in infective juveniles/ml water.

***Effect of pre-wetting period***

There was a significant interaction between the two test dates and corresponding treatments (F = 3.5; df = 4,70; P = 0.011). However, while statistically different, there was no biologically significant difference between the two trials, as all conditions were controlled in the laboratory, and the only varying factor was the nematode inoculum quality which could be ascribed to batch to batch variation. Subsequently, the data for both trials were combined for the one-way ANOVA analysis of percentage codling moth mortality vs. treatment, which showed that the treatments did not differ significantly amongst each other (F = 0.27; df = 4,75; P = 0.9). A slight increase in codling moth mortality was observed, the longer the bins were pre-wet (Figure 1). For the first treatment, when bins were not pre-wet, 65.5 ± 6.5% mortality was obtained. Thereafter, mortality leveled out, ranging between 63.3% and 72.6%.

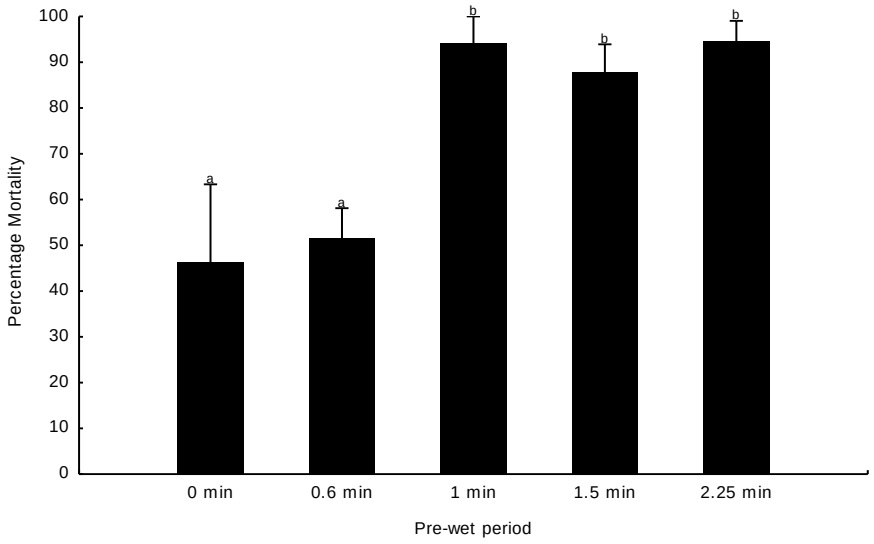


**Figure 1.** Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after submerging minibins in water for different pre-wet time periods before treatment with *Heterorhabditis zealandica* (*SF41*). Different lettering above vertical bars indicates significant differences (one-way ANOVA; F = 0.27; df = 4,75; P = 0.9).

***Effect of humidity***

There was no significant difference in percentage mortality between the two dates (F = 1.82; df = 3,24; P = 0.17). There were however significant differences in percentage codling moth mortality due to nematode infection between all the different humidity treatments which were evaluated (F = 27.06; df = 3,24; P < 0.0001). The higher the level of humidity, the higher the level of mortality was obtained (Figure 2). The highest level of codling moth mortality (88.8 ± 4.9%) was obtained at above 95% RH. A relatively high level of codling moth mortality (63.6 ± 7.3%) was obtained at 75% RH, but below 50% RH and 25% RH, codling moth mortality dropped to 25.9 ± 9.7% and 11.0 ± 4.9%, respectively.

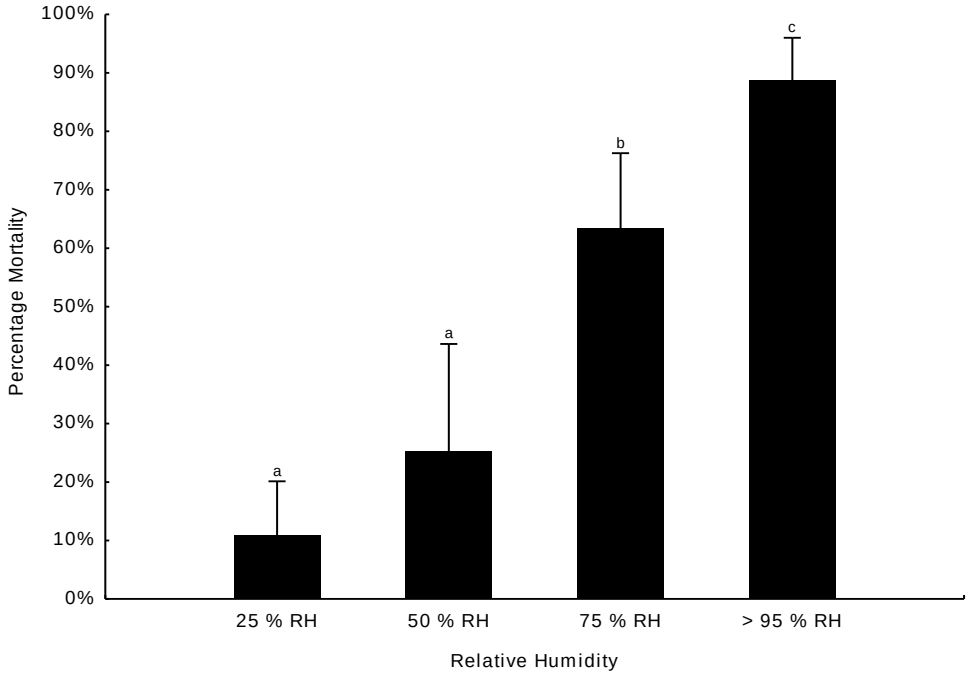


**Figure 2.** Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after exposure to *Heterorhabditis zealandica* (SF41) at different humidity regimes. Different lettering above vertical bars indicates significant differences (factorial ANOVA; F = 1.82; df = 3,24; P = 0.17).

***Effect of incubation time***

There was an increase in mortality as the incubation period lengthened. The highest level of mortality (> 95%) was obtained when larvae were incubated for approximately 153 hours (approximately 7 days) under optimum conditions (> 95% RH and 25°C). The regression of probit mortality on the log of time for the two separate experiments did not differ ($\chi^2$ = 16.98; df = 8; P = 0.030). The regression

equation was Y = 2.87 + 1.74[X], where Y= probit mortality and X = log (time in hours). The $LT_{50,}$ $LT_{90,}$ $LT_{95}$ and $LT_{99}$ values in hours and 95% fiducial limits were 17 (12 – 22), 90 (64 – 148), 146 (96 – 272) and 358 (204 – 875), respectively. The 95% fiducial limits of $LT_{90,}$ $LT_{95}$ and $LT_{99}$ overlapped, demonstrating that beyond 73 hours (approximately three days) after inoculation, larval mortality did not increase markedly.

***Adjuvant Trial***

The addition of adjuvants did not affect nematode survival. No nematode mortality was recorded after exposing IJs to the suspensions containing adjuvants at the recommended dosages for the 24 h period in the laboratory. There was no significant difference in percentage mortality between the two separate test dates (F = 1.64; df = 3,23; P = 0.21. There were no differences in mortality between the two dates (F = 0.41; df = 1,23; P = 0.53), but there were differences between the treatments (F = 8.23; df = 3,23; P < 0.001). Mortality was 27.3 ± 5.0% for the control, 41.3 ± 5.0% for Reverseal 5/1™, 55.2 ± 5.0% for Solitaire™ and 59.3 ± 5.4% for Reverseal 10™. Mortality in the three adjuvant treatments differed from the control, but not between each other (Figure 3).

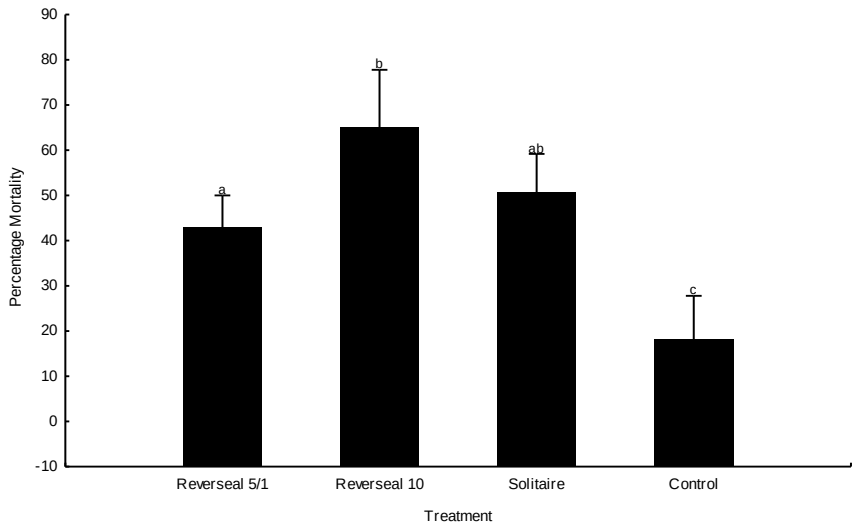


**Figure 3.** Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after exposure to *Heterorhabditis zealandica* (SF41) in four different formulations using Reverseal 5/1™, Reverseal 10™, Solitaire™ and only water. Different lettering above vertical bars indicates significant differences (factorial ANOVA; F = 8.23; df = 3,23; P = 0.0007).

***Tarping Trial***

There were significant interactions between trial test date and treatment (F = 4.29; df = 1,36; P = 0.045). This was due to different ambient conditions, particularly humidity, in which the bins were maintained between the two test trial periods (Trial 1 and Trial 2). For the first trial period, the humidity in the tarped binstack was approximately 90% RH and 25% RH inside the untarped binstack. For the second test date, the humidity inside the binstack was approximately 100% RH and 40% RH inside the untarped binstack. There were differences in mortality between the two test date trials for the mini-bins which were tarped as opposed to the untarped bins (Figure 4). For the first test date during Trial 1, the treatments did not differ significantly from each other (F = 4.99, df = 1,18; P = 0.093), although it should be noted that a much higher level of codling moth mortality was obtained for tarped bins (62.8 ± 5.7%), compared to untarped bins (41.6 ± 7.6%). For the second trial, the same pattern was observed (codling moth mortality for tarped bins 87.7 ± 3.4% and untarped bins 43.3 ± 4.8%), but the difference in codling moth mortality due to nematode infection between tarped and untarped bins was significant (F = 57.95, df = 1,18; P < 0.0001).

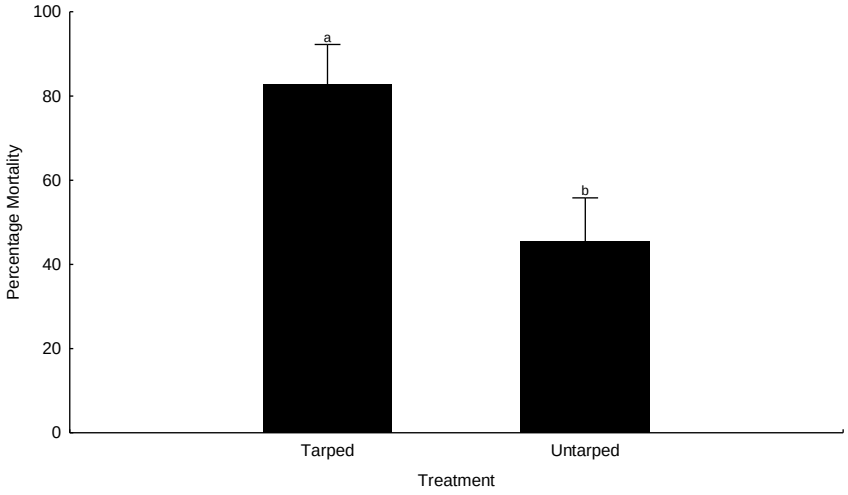


**Figure 4.** Mean percentage mortality (95 % Confidence Interval) recorded for diapausing codling moth larvae after treatment with *Heterorhabditis zealandica* (*SF41*) in tarped or untarped bins. Different lettering above vertical bars indicates significant differences.

## Discussion

The study showed that *H. zealandica* (SF41) had specific requirements fundamental to the success of treating codling moth infested wooden fruit bins. It was important to investigate these factors in the laboratory before commencing with full scale trials, because the data obtained from these preliminary trials will have to form the basis for the operational protocol to be implemented in future trials.

Factors which most influenced the activity of the nematodes were similar to results obtained from previous laboratory bioassays (De Waal 2008; Malan and Addison 2008). An example of this is the concentration of IJs and the subsequent level of codling moth mortality obtained. It was clear from the current study that the nematode concentration was directly proportional to the codling moth mortality. A discriminating dosage was also identified in this experiment [$LD_{50}$ value of 18 IJs/ml (≈ 25 IJs/ml)] (Table 1), for use in laboratory bioassays throughout the rest of this study to evaluate factors such as humidity and moisture effects, whilst achieving adequate kill. This concentration was similar to the 50% mortality value of 13 IJs/ml (Malan and Addison 2008) identified during initial laboratory bioassays. The $LD_{90}$ value when using 187 IJs/ml was a good indication of the concentration which should be applied under operational conditions. This was similar to results from laboratory bioassays by Lacey and Chauvin (1999), Lacey *et al.* (2005) and Malan and Addison (2008), where 100 IJs/ml also resulted in the highest levels of mortality. A similar trend was also observed by Riga *et al.* (2006), where miniature wooden fruit bins infested with oriental fruit moth were treated with two different concentrations (10 and 25 IJs/ml) of *S. feltiae* and significantly higher levels of control were obtained at the higher concentration of 25 IJs/ml tested.

Results obtained for pre-wetting mirrored results from Cossentine *et al.* (2002), suggesting that pre-soaking wooden bins prior to nematode treatments increase efficacy (Figure 1). This could be because pre-wetting lowered the initial water-repellency effect that dry wood naturally has, thereby enabling bins to better absorb inoculum in the subsequent nematode dip. It should be noted that mortality was still recorded for the treatment where these bins were not pre-wet, suggesting that a pre-wet is not necessarily crucial, yet advisable.

Nematodes require a thin water film to maintain activity, and moisture was therefore a major factor directly influencing nematode survival, host location and subsequent infection. Furthermore, it is well known that high humidity is fundamental to successful above-ground EPN application (Wright *et al.* 2005). Our observations during the humidity trial were consistent with this hypothesis, as lower levels of codling moth mortality were obtained at lower humidity, indicating that nematode survival and subsequent efficacy clearly depended on humidity. It was clear that the higher the humidity, the higher the resulting level of larvicidal activity caused by nematode infection. Based on current (Figure 2) and previous results (Lacey and Unruh 1998; De Waal 2008), humidity should be maintained at > 95% RH to ensure maximum codling moth mortality.

Once the required humidity is obtained, it is important to maintain it, because the IJs first need to locate the location of the codling moth larvae in the bin, penetrate the surrounding cocoon and host before the habitat becomes desiccated. A long incubation at optimum humidity is therefore desirable. Results obtained from the incubation trial supported this theory, as mortality increased as the incubation period at optimum conditions (> 95% RH, 25°C) lengthened. The greatest increase in mean mortality occurred during the first day (from approximately 8 to 84% in the first 20 hours), suggesting that during this time, favourable conditions for nematode activity are critical. After approximately three days, mortality leveled off ranging between approximately 90% after 68 hours (approximately three days), 90% after 102 hours (approximately four days) and 94% after 153 hours (approximately six days), suggesting that wooden fruit bins should preferably be maintained at these optimum conditions for at least three days to ensure nematode survival and subsequent infection of codling moth.

One method of overcoming desiccation is to use adjuvants, a key factor influencing nematode survival (Glazer *et al.* 1992). Several studies have shown that the use of adjuvants in aqueous nematode suspensions is beneficial to an above-ground nematode application, as it can increase spray deposition and extend the presence of free water, thereby prolonging the survival and subsequent activity of nematodes (Webster 1973; Glazer 1992; Lacey and Chauvin 1999; Koppenhöfer 2000; Arthurs *et al.* 2004; Lacey *et al.* 2005; De Waal 2008). Adjuvants can also be used to overcome hydrophobicity of especially new wood, thereby enhancing penetration into protected areas of the bins where larvae might reside, such as behind corner supports (Lacey *et al.* 2005). Our results showed that the addition of an adjuvant was beneficial. The highest codling moth mortality (59.3 ± 5.4%) due

to nematode infection was obtained by using Reverseal 10™ for bin treatments (Figure 3). This product contains mixed polymers, which enhance spreader-sticker properties, by increasing the wetability and adhesion of the inoculum on hard to wet surfaces. This is ideal for wooden fruit bin treatments, as it helps to wet the wood and retard evaporation, thereby limiting quick desiccation of IJs. For this reason, Reverseal 10™ seemed to be an appropriate recommendation for addition to formulations.

Tarping wooden fruit bins post-treatment made a significant difference to the humidity inside the bin stacks (Figure 4). In tarped bins during both trials, > 90% RH was recorded, which as previously shown in the humidity trial, was conducive to nematode efficacy. This was also obvious from the higher levels of mortality (Trial 1: 62.9 ± 5.7% and Trial 2: 87.7 ± 3.4%) obtained in the tarped bins in both trials. Low levels of humidity (< 40% RH) in the uncovered bins in both trials caused nematodes to desiccate and lower levels of codling moth mortality was subsequently obtained (Trial 1: 41.6 ± 7.6% and Trial 2: 43.3 ± 4.8%). Tarping wooden fruit bins post-treatment has also previously been shown to significantly increase the level of mortality (Cossentine *et al.* 2002). However, in practice, tarping bins might be too labour-intensive and thus impractical.

Temperature is also one of the key elements in nematode application. It was not evaluated in this particular study, as it has already been documented in literature (Koppenhöfer, 2000). *Heterorhabditis zealandica* has been shown to be active between temperatures of 15 and 30°C, with the optimum temperature for this species ranging between 20 and 25°C (De Waal 2008). This implies that wooden fruit bins will have to be stored at temperatures within this optimum temperature range post-treatment to ensure maximum nematode activity.

In conclusion, the study illustrated that the successful application of *H. zealandica* to disinfest wooden fruit bins of diapausing codling moth larvae is highly dependent on certain conditions and key factors. Should these requirements not be met, the treatment will fail and bins will not be disinfested. Under operational conditions, an application rate of 100 IJs/ml should be sufficient to control codling moth in wooden fruit bins. These bins should be pre-wetted for at least one minute. Post-treatment, bins should be maintained at maximum humidity (> 95% RH) for at least three days to ensure nematode survival and subsequent infection of diapausing codling moth. This can be achieved by tarping bins, or possibly storing them indoors under controlled conditions. Adjuvants (specifically Reverseal 10™)

can be beneficial to an application and can also help overcome detrimental factors such as wood hydrophobicity.

## Acknowledgements

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# CHAPTER 3

## Evaluating mulches together with *Heterorhabditis zealandica* (Rhabditida: Heterorhabditidae) for the control of diapausing codling moth larvae, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae)

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### Abstract

The potential of using an entomopathogenic nematode, *Heterorhabditis zealandica* Poinar, together with different test mulches (pine chips, wheat straw, pine wood shavings, blackwood and apple wood chips) to control diapausing codling moth, *Cydia pomonella* (L.) larvae was evaluated. Mesh cages were identified as a suitable larval-containment method. High levels of codling moth mortality were obtained when using pine wood shavings as mulch (88%) compared to pine chips, wheat straw, blackwood and apple wood chips (41 to 88%). Humidity (> 95% RH) has to be maintained for at least three days to ensure nematode survival. It was also proven that nematodes had the ability to move out of infected soil into moist mulch, to infect the codling moth larvae residing at heights of up to 10 cm. Field experiments showed the importance of climatic conditions on nematode performance. Low temperatures (< 15°C) recorded during the first trial resulted in low levels of control (48%), as opposed to the 67% mortality recorded during the second trial (temperatures ranged between 20 and 25°C). Low levels of persistence (<10 %) were recorded in the mulches post-application. The study conclusively illustrated some of the baseline requirements fundamental to the success of entomopathogenic nematodes together with mulches, for the control of codling moth.

## Introduction

Codling moth, *Cydia pomonella* (L.) is a serious and widespread pest of apples, pears and walnuts (Barnes 1991). Control of this pest is crucial, as in some regions, damage can amount up to a 80% infestation of fruit in orchards if left untreated (Myburg 1980). Previously, codling moth was managed by the use of broad spectrum insecticides (Riedl *et al.* 1998). However, environmental concerns have encouraged the development of alternative pest management tactics, and an area-wide approach to codling moth control is currently being deployed in most successful commercial orchards worldwide, whereby several control measures are integrated, including the use of 'softer' insecticides, attract and kill methods, mating disruption and the sterile insect technique (Blomefield 2003).

The concept of creating a robust orchard environment is linked to the above-mentioned alternative environmentally friendly pest management strategies. To attain this, the direct orchard environment can be manipulated by, for example, diversifying the orchard surface vegetation through mulching (Brown and Tworkoski 2004). Advantages of mulching are well documented and, amongst others, include limiting soil erosion, reducing water loss by evaporation, moderating diurnal fluctuations in soil temperature and increasing soil organic matter content, porosity, water retention and nutrient availability (Matthews *et al.* 2002; Novak *et al.* 2000).

One aspect of mulching in an orchard which has only recently been exploited is the pest management benefits associated with this practice (Brown and Tworkoski 2004). The decomposing mulch material will increase soil biodiversity, which will contribute to soil health (Kennedy 1999) and also further increase the overall diversity in the mulch layer (Matthew *et al.* 2002). Some of these organisms which are present in the mulch are known natural enemies of some of the prevalent pest insects occurring in the orchard and mulching will thus probably increase the efficiency of the natural enemy complex, which in turn will lower the overall pest density in the orchard.

This concept of increasing the natural enemy complex can be achieved by the introduction or augmentation of natural enemies into the mulch when a target insect is also present in the mulch and

at a susceptible stage. In the case of codling moth, a good example of this approach was documented by Matthews *et al.* (2002) where habitat manipulation of the orchard surface by the addition of a mulch to increase ground-dwelling predators and subsequent predation of codling moth, was investigated. A study was also undertaken by Lacey *et al.* (2006b) in which mulch in conjunction with entomopathogenic nematodes (EPNs) were used to help control codling moth which had previously been shown to be very susceptible to infection by EPNs (Kaya *et al.* 1984; Lacey and Chauvin 1999; Unruh and Lacey 2001; Lacey *et al.* 2006a; De Waal 2008).

Lacey *et al.*'s (2006b) study was based on the fact that, when final-instar larvae emerge from fruit in autumn, just before harvest, they search for a suitable overwintering site in which to spin their cocoons. Once they select a site, they spin a cocoon which provides the diapausing larvae with protection throughout the winter period until spring, when the larvae pupate and develop into adult moths. These overwintering sites, such as under loose pieces of bark on trees, in litter at the base of trees or in nearby woodpiles and fruit bins, are normally dry and sheltered (Blomefield 2003; Cossentine *et al.* 2004). Lacey *et al.* (2006b) reasoned that because most of the new apple orchards in the area specific to the study were planted with trellised, high density trees with the smooth bark characteristics of modern varieties on dwarfing rootstocks, the trees offered few cocooning sites for overwintering larvae. Mulch beneath a smooth-bark tree thus provided an attractive alternative habitat for diapausing codling moth larvae and could subsequently be treated with nematodes in order to help manage codling moth.

It has also been proposed that a mulch could enhance the survival and subsequent performance of nematodes (Sweeney *et al.* 1998), as it could act as a buffer against environmental extremes such as direct sunlight, rapid temperature fluctuations and desiccation, known to be the key factors hampering nematode efficacy for above-ground applications (Glazer 1992; Lacey *et al.* 2006a; De Waal 2008).

Few studies have been conducted where nematodes were directly applied on top of a mulch layer to infect a host insect residing in the mulch layer itself. The current study therefore aims to elaborate on this type of approach, specifically for the control of codling moth using *Heterorhabditis zealandica* Poinar in conjunction with different types of mulch in laboratory bioassays and field experiments. The main objective of this study was therefore to identify a suitable containment method for test larvae in mulch, test different mulch types, evaluate nematode behaviour in mulch and assess the performance

of nematodes in mulch in field experiments.

## Materials and methods

### *Source of nematodes and insects*

Infective juveniles (IJs) of *H. zealandica* SF41 (Malan *et al.* 2006) were produced in *Galleria mellonella* (L.) larvae at room temperature. Harvested nematodes were stored horizontally for in 150 ml filtered water in vented 500 ml culture flasks at 14°C and shaken weekly for aeration. One hour before commencing each experiment, IJ concentrations for all trials were quantified in the laboratory in filtered water, using procedures described by Kaya and Stock (1997). Nematodes were used within the first week of harvesting. Codling moth diet and eggs were obtained from the Deciduous Fruit Producers Trust's Codling Moth Rearing Facility located in Stellenbosch, Western Cape Province, South Africa. The colony was periodically infused with field populations. From these eggs, larvae were reared on an artificial diet under diapausing conditions [photoperiod 10:14 (L:D), 25°C, 60% RH]. Fifth instar diapausing codling moth larvae were used for experimentation to avoid larvae pupating during the test period.

### *Bioassay protocol*

All bioassay experiments were conducted in the laboratory at room temperature (25°C). Nematodes in conjunction with mulches were evaluated in plastic containers (19 x 15 x 8 cm for the retrieval technique experiment) and (13 x 13 x 7 cm for the mulch type experiment). These containers were filled with 2 cm of loamy sand orchard soil (10% Clay, 4% Silt, 86% Sand, pH = 4.5), collected from a Forelle pear orchard on the experimental farm, Welgevallen, located in Stellenbosch, Western Cape Province, South Africa. Containers were placed in a freezer at -10°C two days before the trial was conducted. Containers were then removed from the freezer, 24 h before commencing each trial. The appropriate mulch specific to the trial being conducted was applied on top of the soil to a depth of 5

cm. Monitoring cages were prepared prior to the trial and buried 2 cm below the mulch surface on the day of the trial. These cylindrical monitoring cages were based on the design used by Duncan *et al.* (2003). Each cage comprised a 40-mesh stainless steel cylinder (7 x 3 cm diameter), fitted at each end with polypropylene caps. On the day before each trial, cages were filled with the specific mulch being tested, and 20 diapausing codling moth larvae were added to each cage and allowed to spin into cocoons between the mulch pieces over a 24 h period. All nematodes and water applications were made using calibrated shoulder pump sprayers (Dal Degan, Italy). Previous laboratory experimentation, where SF41 *H. zealandica* was inoculated directly onto codling moth larvae, indicated the $LD_{90}$ value to be approximately 20 IJs/$cm^2$ (De Waal 2008) and this concentration was therefore used for most of the trials in the current study in either 500 ml (in the retrieval technique experiment) or 200 ml (in the mulch type experiment) of water per container. In the case of the control treatments, the same volume of water only was applied to each container. After treatment, containers were closed with their lids to help maintain > 90% RH (monitored by inserting Hobo® H8 Pro Series data logger into the containers) throughout the trial period and were then incubated in the dark at 25°C for seven days. After incubation, cages were removed from the plastic containers and codling moth larvae were taken out of the cages for mortality assessments. Larvae were dissected in Ringer's Solution to confirm nematode infection.

***Codling moth retrieval technique***

For the codling moth retrieval technique experiment, the standard described bioassay protocol was followed using pine wood shavings as mulch. Two other methods, where test larvae were added to the mulch in addition to the mesh cages as previously described, were also tested. The different larval containment/retrieval methods which were evaluated in this trial were mesh cages, cardboard strips and uncontained larvae (Image 1). For the first containment/retrieval method, mesh cages (each filled with pine wood shavings and 10 diapausing codling moth larvae) were prepared. The second containment/retrieval method consisted of perforated double-fluted cardboard strips, which were prepared as described by Lacey and Unruh (1998), where cardboard strips (8 x 1.9 cm) were cut and placed in 9 cm diameter Petri dishes. These strips were perforated beforehand, using a tailor's pattern marker with approximately 75 holes (< 0.5 mm diameter) on each side to allow passage of IJs. Ten diapausing larvae were placed in each Petri dish in well-lit conditions at room temperature and

allowed to spin cocoons in the cells of the cardboard over a 24 h period to encourage larvae to enter the flutes. For the third technique, uncontained larvae were used. Ten larvae were added to the mulch 24 h prior to the nematode application to allow them to spin cocoons in the mulch itself. A total of 22 containers were prepared as described in the standard bioassay protocol, each containing one mesh cage, one perforated cardboard strip and 10 freely added larvae. Of these 22 containers, 11 were treated with water as control treatments and 11 were treated with nematodes. The entire experiment was repeated on two separate dates.

**Image 1.** The different larval containment/retrieval methods which were evaluated in the codling moth retrieval technique experiment, including (from left to right) uncontained larvae in mulch substrate, a mesh cage (7 x 3 cm) and a perforated cardboard strip (8 x 1.9 cm).

***Effect of mulch type***

Five different types of mulch (pine wood *Pinus radiata* D. Don. chips and shavings, wheat straw *Triticum aestivum* L., blackwood *Acacia mearnsii* De Wild and apple wood chips *Malus domestica* Borkh.) were evaluated (Image 2), using the described bioassay procedure and nematode concentration of 20 IJs/cm$^2$. Twenty plastic containers (ten treated with nematodes and ten treated with water as a control) were prepared for each of the five mulch types tested. One mesh cage, containing ten diapausing codling moth larvae, was added to each container. The experiment was repeated on two separate dates.

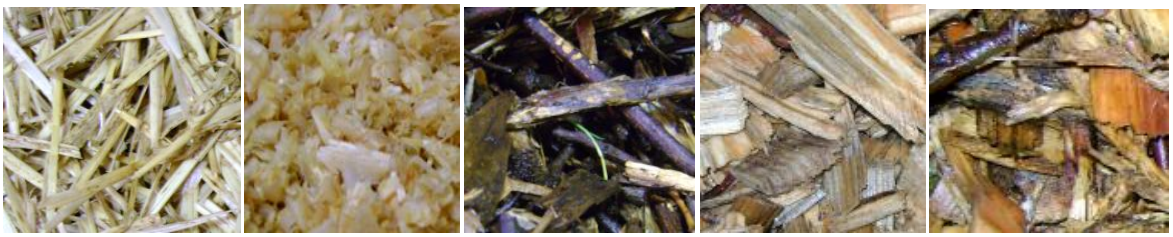

**Image 2.** The different mulches tested in the mulch type experiment included (from left to right) wheat straw, pine wood shavings, blackwood, pine wood chips and apple wood chips.

***Effect of incubation time***

To determine how long cages should be left in a moist mulch (> 95 % RH) to ensure infectivity, the standard bioassay protocol was followed, using eight large cuboidal plastic containers (50 x 26 x 15 cm), filled with 5 cm soil and 10 cm mulch (either straw or apple wood chips). A Hobo H8 Pro Series data logger (Onset Computer Corporation, Massachusetts) was placed in the mulch of each container to monitor humidity throughout the trial period. Ten mesh cages were added to each container, each cage filled with 20 diapausing codling moth larvae and the relevant mulch. Of the four containers prepared for each mulch type being tested, two were sprayed with nematodes and two with water to serve as control treatments. Two cages were removed each time from containers on pre-determined time intervals (4, 6, 9, 13.5, 20.3, 30.4, 45.6, 68.3 and 102.5 h). Before closing the lid of the container, a black plastic bag was placed on top of the mulch to further help maintain moisture. Removed larvae were then rinsed with filtered tap water to remove surface nematodes and placed in Petri dishes lined with moistened filter paper to allow nematode development for another five days. Mortality was assessed as previously described. The experiment was repeated on two separate dates.

***Upward movement of infective juveniles***

Pine wood shavings were used as test mulch to verify whether nematodes had the ability to move upwards out of the soil into the mulch to seek out diapausing codling moth larvae residing at different heights. Three heights were investigated (0, 5 and 10 cm) and for each height, twenty cylindrical plastic containers (14 cm diameter, 11 cm tall) were filled with 1 cm orchard soil. *Heterorhabditis zealandica* IJs were inoculated onto the soil surfaces of half of the containers at a concentration of 20

IJs/cm$^2$ in 5 ml of water, and the remaining containers were treated only with 5 ml of water to serve as control treatments. Containers were filled to the top with moistened pine wood shavings. To position codling moth larvae in the mulch at the specific height being tested, larvae were confined to 0.75 ml pierced Eppendorf tubes (Kehres *et al.* 2001). Four tubes with one larva each were placed at each of the heights being investigated. The containers were then incubated at 25°C in the dark for seven days. After incubation, codling moth larvae were removed from the tubes and, mortality was assessed. The experiment was repeated on two separate dates.

***Field applications***

The performance of *H. zealandica* under field conditions in test mulches (wheat straw and apple wood chips) was evaluated in a Forelle pear orchard on the experimental farm Welgevallen, located in Stellenbosch, Western Cape Province, South Africa. A completely randomized block design was used for the experimental layout, with four rows, each containing eight treatment trees, with three buffer trees between each treated tree and two buffer rows separating treatment rows. Treatments were: (1) straw with nematodes, (2) straw with water, (3) chips with nematodes and (4) chips with water. A 20 cm thick test mulch layer was applied underneath each treatment tree in a 1 m wide band around the tree stem (Image 3).

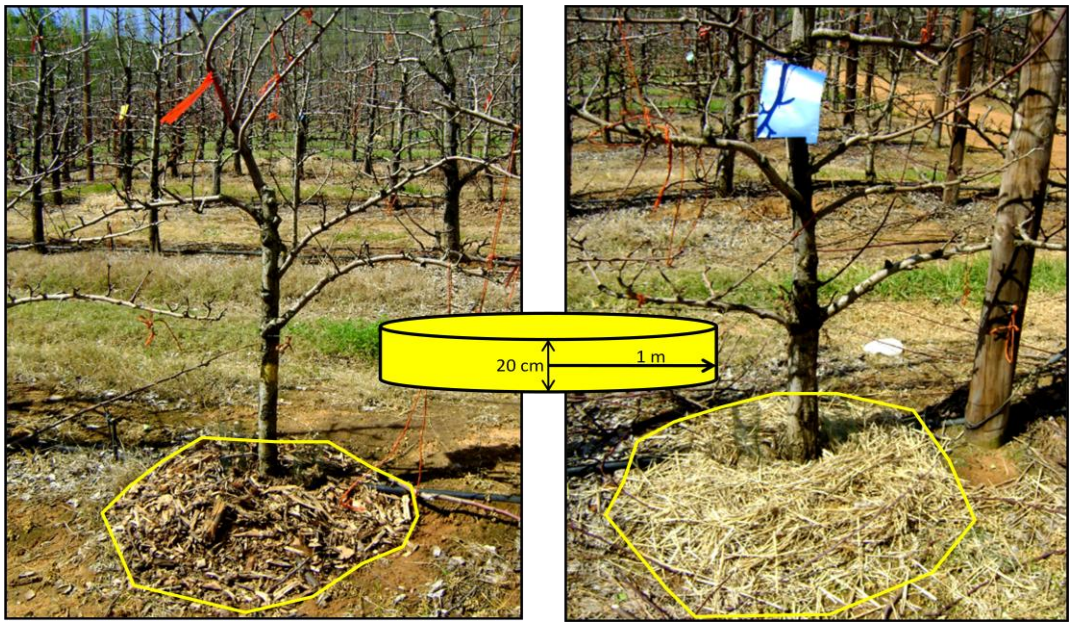


**Image 3.** Test mulches were applied in a 20 cm thick layer around each treatment tree in a 1 m band around each stem.

Half of the 32 treatment trees received wheat straw (Treatments 1 and 2) as test mulch and the remaining half received apple wood chips (Treatments 3 and 4). Both trials were conducted just after sunrise during autumn 2009 (Trial 1: 3 September 2009 and Trial 2: 19 October 2009). Different trees were used for the two trials to prevent contamination. On the day of each trial, two mesh cages (filled with the test mulch and ten diapausing codling moth larvae) were buried in the test mulch 5 cm below the mulch surface at each treatment tree. One hour before nematode treatment applications, mulches were thoroughly pre-wetted, using shoulder pump sprayers (Dal Degan, Italy). Suspensions of IJs at a concentration of 20 IJs/cm² in 1 L water and Solitaire™ (polyether-polymethylsiloxane co-polymer / vegetable oil (EW) Safagric) at a concentration of 1 ml Solitaire™/Lℓ water were applied as wetting agent. Control plots (Treatment 2 and 4) were treated with 1 L of water and Solitaire™. Cages were removed from the mulches seven days after nematode application and taken back to the laboratory for assessment. Larvae were removed from the cages for mortality assessment and dissected to confirm nematode infection. If infection was detected in a control plot, a single female was removed from the insect cadaver for species verification. This was done using molecular techniques as described by Nguyen (2007) for the DNA extraction, amplification of the ITS region and final sequencing reactions using the primers: TW81 and AB28 (Hominick *et al.* 1997). BioEdit version 7.0.4 (Hall 1999) was used for sequence editing, verifying base calls and obtaining the final consensus sequence. This sequence was then aligned with *H. zealandica* SF41's sequence (EU699436) using the ClustalW multiple alignment function (Thomson *et al.* 1997).

Weather data were downloaded from the Helderberg Weather Station in Stellenbosch for each trial date and one Hobo® H8 Pro Series data logger was buried in each of the mulch types to monitor temperature and humidity, and put on the scaffold branches of a tree in the middle of each treatment row to monitor temperature and humidity above mulch level in the orchard on the day of each field application.

In both field trials, the residual activity of the nematodes in mulches was investigated seven days post-application. Two 1 L plastic containers were filled with apple wood chips and straw which was collected underneath each treatment tree to assess residual activity, by looking at the presence and infectivity and thereby persistence of the nematodes in the mulch. This was assessed in the laboratory using the insect baiting technique (Bedding and Akhurst 1975) which would confirm

whether the nematodes were still active and infective and Cobb's Extraction method (Cobb 1918) which would confirm the presence of the nematodes in the mulch. For the insect baiting technique, 500 ml plastic containers were filled with each of the collected mulches and ten diapausing codling moth larvae were added as trap insects, covered with a lid and placed in a growth chamber for seven days at 25°C. Thereafter, mortality was assessed and larvae were dissected to confirm infection. Using Cobb's Method, the resulting clean water samples obtained after rinsing the mulches which contained various types of nematodes were transferred to 100 ml glass cylinders, and nematodes were allowed to settle at the bottom of the cylinders. A 1 ml droplet of the resulting concentrate was inoculated onto a Petri dish (85 mm diameter) lined with filter paper and 10 diapausing codling moth larvae were added to each dish. Petri dishes were placed in plastic containers lined with moistened filter paper and incubated at 25°C for seven days, whereupon the mortality and infection rates were assessed as previously mentioned.

***Statistical analyses***

Data obtained from the retrieval technique evaluation, upward movement of nematodes experiment, mulch type experiment and field application were analyzed using a factorial ANOVA with trial test date and relevant treatments as separate factors, using Statistica 9.0 software (Statsoft Inc. 2009). The short term persistence data was also analyzed using a factorial ANOVA, but with test date, extraction method, treatment and mulch type as factors. If there were no significant trial test date versus treatment interactions, it meant that treatments responded consistently over the two trials that were conducted, and therefore the data from the two trials were combined and a one-way ANOVA was done on the percentage mortality vs. treatment. If residuals were not normally distributed, the main effects were tested with Kruskal Wallis tests (Kruskal and Wallis 1952). In the case of the field application data, the trials were analyzed separately using one-way ANOVAs as there were climatic differences between the trials and data could therefore not be pooled in order to determine whether there were significant differences between the treatments. Interaction effects which were significant were analyzed with Bonferroni's method or Bootstrap multiple comparisons (Efron and Tibshirani 1993), depending on whether residuals obtained in the ANOVA were normally distributed or not. For the retrieval technique evaluation and mulch type experiment, Abbott's correction factor (Abbott 1925) was used to correct control mortality at each of the treatments, and the resulting corrected mortality

was then used for the final ANOVA analysis. To statistically evaluate the incubation-period experiments, a Probit analysis was conducted using NCSS (Hintze 2007). For the Probit model, the number of control replications for each treatment had no influence on the results of the analysis, because all the controls were lumped together, as were all the replications, for each treatment during the analysis. The $LD_{50, 90, 95, 99}$ values were estimated with their corresponding 95% fiducial limits. Means ± SE are presented throughout the text.

## Results

### *Codling moth retrieval technique*

High levels of codling moth mortality (> 89%) were recorded for all three retrieval techniques, which also did not differ significantly from each other ($F = 1.61$; $df = 2,61$; $P = 0.21$). The highest level of larval mortality due to nematode infection was obtained for larvae placed in cardboard strips (96.2 ± 2.99%), followed by larvae which were placed in mesh cages (90 ± 2.99%) and larvae which were released freely in the mulch (89 ± 2.99%) and larvae. The percentage retrieval of codling moth larvae from mulch was proportionally higher for the cages (93%), as opposed to the strips (19%) and the freely-added larvae (81%).

### *Effect of mulch type*

Treatments differed significantly ($F = 4.29$; $df = 4,40$; $P = 0.006$) as varying levels of codling moth larvicidal activity were recorded in the five different mulch types (pine chips, wheat straw, pine wood shavings, blackwood and apple wood chips) (Figure 1) tested. Significantly higher levels of mortality was recorded in the pine wood shavings (88 ± 5.05%), than in the blackwood (72 ± 5.05%), pine chips (67 ± 5.05%), apple wood chips (41 ± 5.05%) and straw (31 ± 5.05%) treatments.

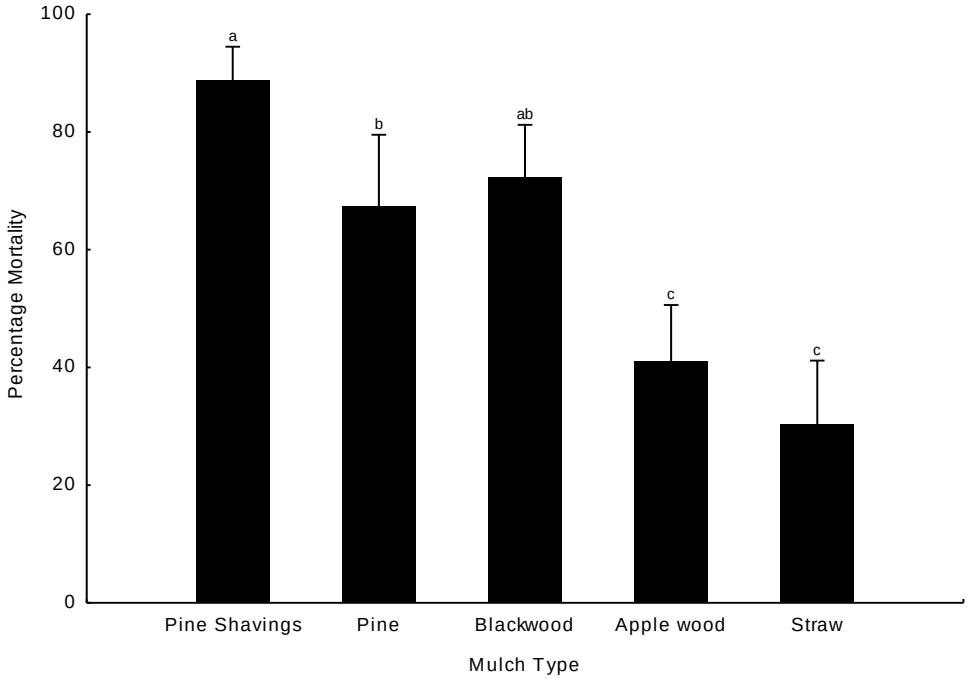


**Figure 1**. Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after exposure to *Heterorhabditis zealandica* (SF41) in different mulch types (pine chips, wheat straw, pine wood shavings, blackwood and apple wood chips). Different lettering above vertical bars indicates significant differences (factorial ANOVA; $F = 21.25$; $df = 4,96$; $P < 0.001$).

***Effect of incubation time***

Humidity remained above 95% in all the containers throughout the trial period. In both types of mulch (apple wood chips and wheat straw) tested, there was an increase in codling moth mortality due to nematode infection as the incubation period lengthened (Figure 2).

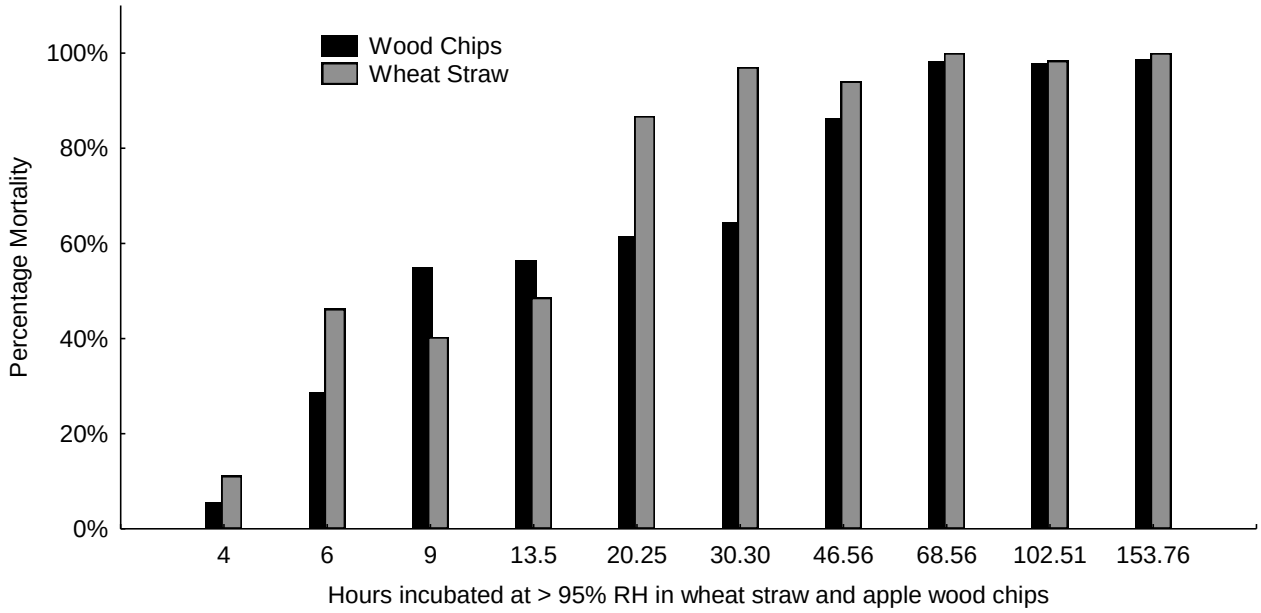


**Figure 2.** The effect of incubation time at > 95% RH in either apple wood chip mulch or a wheat straw mulch on the average codling moth mortality rate recorded after exposure to *Heterorhabditis zealandica* (SF41).

Confidence intervals of 95% overlapped for both trials for the apple wood chips (Trial 1: 1.99 - 2.94 and Trial 2: 1.75 – 3.01) and the wheat straw (Trial 1: 1.87 - 2.87 and Trial 2: 1.93 – 2.89), resulting in independent probit regression lines being the same for both Trial 1 and Trial 2 and data could therefore be pooled for analysis. The regression formulae were Y = 2.51 + 2.2[X] for apple and Y = 2.56 + 2.52[X] for straw, where Y = probit mortality and X = log (time in hours). For the straw treatment, the $LT_{50}$, and $LT_{90}$ values in hours (and 95 % fiducial limits) were 9 (8 – 11) and 30 (25 – 36), respectively (Table 1). The fact that the 95% fiducial limits of $LT_{90}$ (25 – 36), $LT_{95}$ (34 – 52) and almost $LT_{99}$ (58 – 104) overlapped to a certain degree, indicates that beyond the last-mentioned intervals, maintaining optimum conditions will not necessarily increase the level of mortality obtained, as the biggest increase in mortality already occurs during the first day. Mortality still increases thereafter, but at a steady rate for each incremental increase. Almost the same pattern was observed for the apple wood trial and the same can therefore be deduced for the straw trial, where $LT_{50}$, and $LT_{90}$ values in hours (and 95 % fiducial limits) were 13 (11 – 15) and 50 (40 – 61) respectively (Table 1) and 95% fiducial limits of $LT_{90}$ (40 – 61), $LT_{95}$ (56 – 93) and almost $LT_{99}$ (103 – 208) almost overlapped.

**Table 1.** LT-values (hours) and their corresponding 95% fiducial limits obtained from the trial investigating the effect of different exposure periods of codling moth larvae in either apple wood chips or straw to *Heterorhabditis zealandica* (SF41).

| LT | Value | 95 % Lower fiducial limit | 95 % Upper fiducial limit |
|---|---|---|---|
| | | Wheat Straw | |
| 50 | 9 | 8 | 11 |
| 90 | 30 | 25 | 36 |
| 95 | 42 | 34 | 52 |
| 99 | 78 | 58 | 104 |
| | | Apple Wood Chips | |
| 50 | 13 | 11 | 15 |
| 90 | 50 | 40 | 61 |
| 95 | 72 | 56 | 93 |
| 99 | 147 | 103 | 208 |

***Upward movement of infective juveniles***

There was a negative correlation between the heights at which the codling moth larvae were placed and the percentages of codling moth mortality obtained at each height, as the larvacidal activity significantly decreased as the height at which the codling moth were placed in the mulch increased ($F = 71.57$; 2,54; $P < 0.001$). The highest mortality rate was recorded when larvae in tubes were placed directly on the soil surface (100%). Thereafter, larval mortality decreased to 90 ± 5.8% at 5 cm and 34 ± 5.8% at 10 cm.

***Field applications***

There was no significant interaction between the trials and corresponding treatments. However, there was a significant difference between the level of codling moth mortality between the two dates ($F = 7.9$; df = 1,56; $P = 0.007$). Treatments differed significantly from each other for both Trial 1 ($F = 29.6$;

df = 3,28; P < 0.0001) and Trial 2 (F = 27.1; df = 3,28; P <0.0001). Higher levels of codling moth mortality regarding nematode treatments were obtained in apple wood chips for both trials (Trial 1: 61.8 ± 5.49 %; Trial 2: 77,9 ± 7.04 %), compared to that of wheat straw (Trial 1: 33.9 ± 5.49 %; Trial 2: 56.7 ± 7.04 %) (Figure 3). The average level of codling moth mortality for the nematode treated plots was higher in Trial 1 (48 %) than in Trial 2 (67 %). There were climatic differences between the two trials, as summarized in Table 2. For Trial 2, the 4 % and 7 % nematode infection recorded in the two control treatments (Treatment 2 and 4) was confirmed to be *H. zealandica*, as the sequences obtained from the nematodes isolated from the infected insect cadavers aligned perfectly with the SF41 sequence of *H. zealandica*.

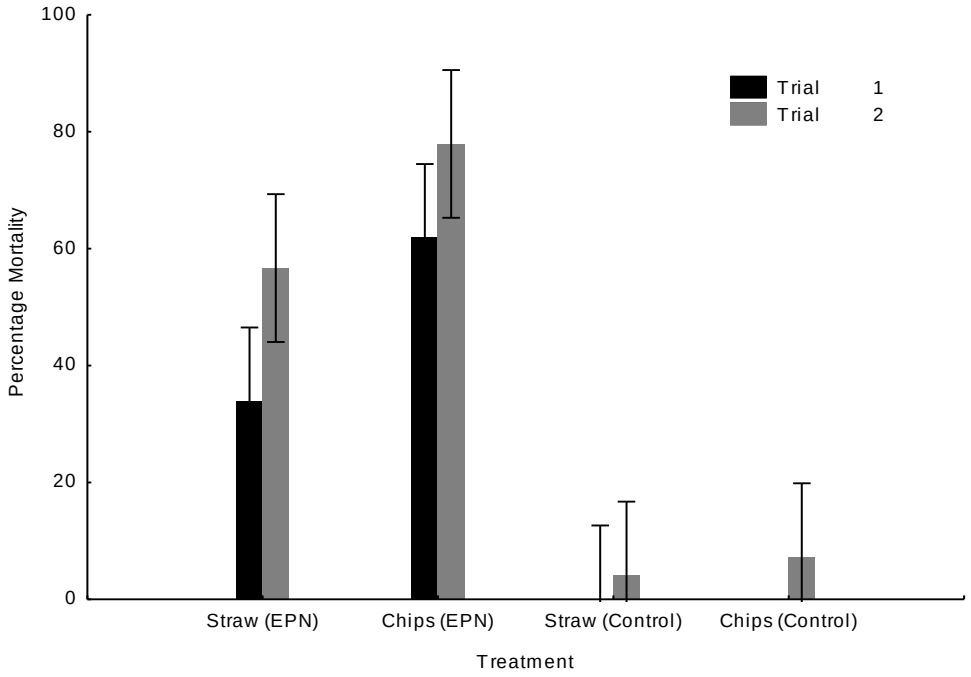


**Figure 3.** Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after exposure to *Heterorhabditis zealandica* (SF41) in wheat straw and apple wood chips during two field trials conducted in September 2009 (Trial 1) and October 2009 (Trial 2) (factorial ANOVA; F = 0.91; df = 3,56).

For the short term persistence analysis, all three- and four-way interactions were insignificant. Among the two-way interactions, only the trial test date versus baiting method interaction was significant (F = 4.54; df = 1,11; P = 0.035). There was no significant difference between the different mulch types in which the residual activity of the nematodes was tested (F = 4.54; df = 1,11; P = 0.035), but the

treatments differed significantly from each other (F = 7.15; df = 1,11; P = 0.0086). Higher levels of larvicidal activity were recorded in the nematode treated plots for Trial 1 (11.23 ± 3.59%) than for Trial 2 (6.51 ± 3.59%). Similar levels of larvacidal activity were recorded for both methods of nematode recovery (eg. Trial 1 insect baiting technique: 11.23 ± 3.59% vs. Cobb's method: 11.66 ± 3.59%). Mortality recorded in the nematode treated straw plots was higher (19.62 ± 3.59%) than levels recorded in the apple wood chip plots (9.58 ± 3.59%).

## Discussion

The study indicated that the orchard agroecosystem could be manipulated to the advantage of *H. zealandica* to ensure the successful reduction or elimination of diapausing codling moth larvae in a moistened mulch layer. This was facilitated in the field by enhancing nematode survival and subsequent efficacy by the addition of a moistened mulch layer, coupled with favourable nematode application techniques. However, prior to the field application, several factors fundamental to the eventual success of this particular trial were first investigated in the laboratory.

High codling moth mortalities of above 89% were obtained for all three methods (cages, strips and adding the larvae uncontained to the mulch) evaluated, indicating that nematodes could easily penetrate through the cages, strips and cocoons of larvae placed separately in the mulch to infect and kill codling moth larvae. This suggests that all three methods are suitable for use as a containment option for test larvae. However, regarding the retrieval rate, there were notable differences between the methods. Larvae escaped from the open cell edges of the cardboard strips, resulting in a very low recovery rate of 19 %. A lower recovery rate of 81% was also observed for the larvae which were allowed to spin up naturally in the mulch layer, incorporating pieces of mulch into their cocoons which camouflaged them and made it difficult to find them after the trial period. Larvae could not easily escape from cages, so few insects were lost throughout the trial period (93% recovery). Mesh cages have previously also been used with great success in trials as a containment-method of test insects during nematode applications onto soil (McCoy *et al.* 2000; Duncan *et al.* 2003). Based on the current study's results and previous success with these cages, the method was subsequently selected as the

most suitable containment- and retrieval method for codling moth larvae in mulch, and for this reason used in proceeding experiments.

The highest level of codling moth mortality was obtained when pine wood shavings were used as mulch, compared to pine chips, wheat straw, blackwood and apple wood chips. This might be attributed to smaller particle size of pine wood shavings as opposed to the other mulch types, which would allow for better moisture-retention and subsequent nematode performance. Moderate levels of control were nevertheless obtained from other mulches, suggesting that these mulches were also suitable for use. Of these, wheat straw and apple wood chips are readily available to most local growers, and therefore the most practical option for future use. These two mulches were consequently used for the rest of the study. Blackwood, pine chips and pine wood shavings were included in the mulch type experiment, as these substrates are also sometimes used in local orchards as mulches. Lacey *et al.* (2006b) also compared different mulch types (clover, shredded paper, grass hay and wood chips) for their utility to enhance the efficacy of nematodes applied against diapausing codling moth larvae in the field. In relation to the current study, a similar trend in comparable larvacidal activity levels was obtained between their tested mulches, where cardboard strips were used for test-insect containment. *Steinernema carpocapsae* was inoculated onto test plots at a concentration of 2.5 x $10^9$ IJs/ha. Resulting codling moth mortality was lower in wood chips (76 - 79%) and clover plots (76-79 %) relative to paper, grass hay and bare plots (93 - 97%) (Lacey *et al.* 2006b).

The trial investigating incubation time, gave a good indication of how long high levels of humidity (> 95% RH) should ideally be maintained in apple wood chip- and straw-mulch to prevent nematode desiccation and ensure survival. The results obtained indicated that apple wood chip mulch has to be kept wet for at least 3 days and straw mulch for approximately 2 days to achieve 95% mortality. However, in the field, other external factors, such as wind can also influence the rate at which the substrate dries out. To ensure optimum kill it is therefore advisable to maintain high moisture levels in the mulch for a longer period of time as suggested. Previous work conducted with *H. zealandica* indicated that only 5 hours of high humidity were required to achieve 95% codling moth larval mortality. This, however, was when nematodes were applied directly onto the target insect as an above-ground application on trees (De Waal 2008). When codling moth larvae are located in a more cryptic habitat, such as in mulch, a longer search period under optimum conditions have to be maintained to guarantee that the nematodes locate and successfully penetrate the host insect before

the habitat becomes desiccated (Lacey *et al.* 2006).

Maintaining high levels of humidity in mulch is fundamental to the success of a nematode treatment, as it will prevent nematodes from desiccating (Wright *et al.* 2005). This can be facilitated by wetting the mulches using applicable irrigation-options or by applying the nematodes during rainy weather. There is, however, the risk of over-irrigation or rainfalls being too heavy, which would subsequently wash the nematodes off into the soil. Results recorded from the trial evaluating the ability of *H. zealandica* IJs to move out of the soil and up into the moistened mulch layer to successfully infect codling moth larvae placed at different heights (0, 5 and 10 cm), indicate that *H. zealandica* has the ability to move back up into the mulch after being washed into the soil by heavy rains or over-irrigation. It has been noted that *H. zealandica* has a high innate dispersal rate in soil (Koppenhöfer and Fuzy 2008). *Heterorhabditis* generally utilizes a cruising strategy to locate their hosts (Susurluk *et al.* 2003), but no reference has been made about this species' ability to move upwards out of the soil into a mulch. Results obtained from this study are thus very promising, as codling moth mortality due to nematode infection was recorded from up to 10 cm above soil surface level in pine wood shavings.

It has previously been demonstrated that EPNs have specific climatic requirements fundamental to the success of field applications aimed at targeting diapausing codling moth larvae residing in cryptic habitats such as on trees (Lacey and Unruh 1998) and in mulches (Lacey *et al.* 2006b). Of these environmental conditions, optimum moisture and moderate temperature has been underlined as being the most crucial requirements for most species of EPNs (Kaya and Gaugler 1993), including *H. zealandica* (De Waal 2008) to ensure survival and subsequent success in the field. In the current study, field trials are aimed at evaluating the performance of *H. zealandica* in different mulch types under varying climatic conditions. Vast differences in climatic conditions were recorded during the two trial periods. Theoretically, ambient conditions (moderate temperatures and high levels of humidity in mulches) were more favourable to EPN survival and therefore performance during Trial 2 than in Trial 1, which explains the higher levels of codling moth mortality recorded for the aforementioned Trial 2 (Figure 3). The low temperatures (< 15°C) prevailing throughout Trial 1's experimental period clarified the lower levels of control obtained, compared to the higher levels of mortality obtained in Trial 2, where average temperatures were within the optimum temperature range (20 - 25°C) previously determined for *H. zealandica* (De Waal 2008). Excessive rain recorded throughout Trial 1's experimental period, could further explain the lower levels of mortality obtained for Trial 1 as opposed

to Trial 2, due to nematodes possibly being washed off mulches. Differences amongst the levels of mortality obtained between the different mulch types tested, could be ascribed to wood chips being better able to absorb moisture and thus easily able to maintain higher levels of humidity which is crucial to nematode survival (Koppenhöfer 2007), as opposed to wheat straw, which tends to dry out easily.

Contamination in the control plots for Trial 2 was suspected due to the perfect alignment of SF41 *H. zealandica*'s sequence to the sequence generated from the contamination sites, and could be linked to human activity, wind drift or contaminated spraying equipment. Another possible explanation could be the natural presence of an isolate of *H. zealandica*. This could however not be confirmed, as no baseline sampling was done prior to the trial.

Lacey *et al.* (2006b) conducted an experiment illustrating the short-term persistence/residual activity of nematodes in mulch, three days post-application. Similar results were obtained in the current study, but lower levels (< 10%) of codling moth mortality were recorded, as opposed to the Lacey *et al.*'s (2006b) study with 12 - 17% residual larvicidal activity after three days. Nonetheless, the same statement made by Lacey *et al.* (2006b) is valid, suggesting that a longer exposure time of codling moth larvae to nematodes results in higher levels of infection, and that any level of residual activity recorded for nematodes in mulch post-application will thus be conducive to the success of an application. This concept has also previously been demonstrated for *H. zealandica* during an incubation time trial with codling moth larvae as test insects in the laboratory, showing that the level of mortality increased as the time period of nematode exposure lengthened (De Waal 2008).

In conclusion, the study illustrated some of the important baseline requirements which are fundamental to the successful use of nematodes in conjunction with mulches for the control of diapausing codling moth larvae. Accordingly, these results can assist in developing a framework for the use of EPNs against codling moth in an orchard agroecosystem as part of an integrated pest management programme.

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# CHAPTER 4

## Efficacy of entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae) against codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae) in temperate regions*

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### Abstract

The biocontrol potential of South African isolates of *Heterorhabditis zealandica*, *Steinernema citrae*, *S. khoisanae*, *S. yirgalemense* and *Steinernema* sp., was evaluated against codling moth, *Cydia pomonella*. Codling moth was susceptible to all six nematode isolates at a concentration of 50 infective juveniles/insect (78 - 100% mortality). Low temperatures (10h at 17°C; 14h at 12°C) negatively affected larvicidal activity (≤ 3%) for all isolates. All tested isolates were most effective at higher levels of water activity ($a_w = 1$). The average $a_{w50}$-value for all isolates tested was 0.94 (0.93 - 0.95), except *S. khoisanae* 0.97 (0.97 - 0.98). Regarding host-seeking ability, no positive attraction to host cues could be detected amongst isolates, except for *H. zealandica*. Three of the isolates, *H. zealandica, S. khoisanae* and the undescribed *Steinernema* sp., were selected for field-testing and proven to be effective (mortality > 50%). Insect containment methods used during field experimentation was shown to influence larvicidal activity, as different levels of mortality were obtained using various containment methods (wooden planks vs. pear tree logs vs. mesh cages). Pear tree logs were impractical. Predictive equations were subsequently developed, enabling future trials to be conducted using either planks or cages, enabling the prediction of the expected level of control on tree logs. All tested isolates therefore showed a certain degree of biological control potential, however, none of the experiments showed clear efficacy-differences amongst isolates. The study highlighted

the importance of environmental factors to ensure the successful application of these nematodes for the control of diapausing codling moth larvae in temperate regions.

## Introduction

The codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), is a major pest of pome fruits throughout temperate regions of the world. It has the ability to complete up to four generations per growing season if left untreated (Barnes 1991). Adult moths emerge from pupae in late spring and lay their eggs individually on the fruit and adjacent leaves. Neonate larvae hatch from eggs and move towards the fruit, where they penetrate into the fruit to feed, creating a visible frass-filled tunnel as they feed. This action and its resultant appearance reduce the market value of the crop (Welter 2008). Final instar larvae then exit from the fruit in search of a cryptic habitat mainly on trees, where they either cocoon and pupate, or spend the winter as diapausing larvae (Blomefield 2003).

Previously, codling moth control involved the extensive use of broad-spectrum insecticides (Giliomee and Riedl 1998). However, concerns over human safety, environmental impact, widespread dispersal of resistant populations of codling moth and the sustainability of synthetic pesticides in agroecosystems, has encouraged the development and use of alternative pest management technologies (Blomefield 2003), such as the use of entomopathogenic nematodes (Kaya *et al.* 1984; Lacey and Unruh 1998; Unruh and Lacey 2001; De Waal 2008).

Entomopathogenic nematodes in the Families, Steinernematidae and Heterorhabditidae, are biological control agents (Adams and Nguyen 2002). These nematodes are able to kill insects with the aid of a mutualistic bacterium (*Photorhabdus* spp. for heterorhabditids and *Xenorhabdus* spp. for steinernematids), which is carried inside their intestine (Boemare 2002). The nematodes generally complete two to three generations within the host, after which a non-feeding and developmentally arrested third-stage dauer juvenile emerges to seek new hosts (Adams and Nguyen 2002). This stage is generally referred to as the infective juvenile (IJ) stage and they enter host insects through natural openings such as the mouth, anus and spiracles or occasionally even through the cuticle, whereupon

the previously mentioned bacteria is released inside the insect, which would then normally kill the insect within 48 h (Poinar 1990; Dowds and Peters 2002). Although entomopathogenic nematodes are generally pathogenic to a wide variety of insect pests [e.g., some nematode species can infect more than 100 different hosts in the laboratory (Poinar 1979)], successful commercialization and application on a commercial basis has been limited to relatively few target insects (Grewal and Georgis 1999; Shapiro-Ilan *et al*. 2002).

Codling moth is susceptible to entomopathogenic nematodes (Kaya *et al.* 1984; Lacey *et al.* 1998; Lacey and Chauvin 1999; Vega *et al.* 2000; Unruh *et al.* 2001; Cossentine *et al.* 2002; Lacey *et al.* 2005; Lacey *et al.* 2006a; Lacey *et al.* 2006b; Lacey *et al.* 2007), and is one of the few examples where entomopathogenic nematodes are used on a commercial basis to help control an insect pest. The developmental stage of codling moth best suited for control with entomopathogenic nematodes is the diapausing larval stage, which occurs between late summer and early spring in cryptic habitats, such as underneath loose pieces of bark or in pruning wounds on trees (Lacey *et al.* 1998). Eliminating this stage would protect fruit from damage in the following growing season (Lacey *et al.* 2007). Cryptic habitats have also been noted to be favourable environments for entomopathogenic nematodes, as they are shielded from environmental conditions which might be harmful to the nematodes, such as low levels of humidity and direct exposure to sunlight (Lacey *et al.* 1998; Lacey *et al.* 2007).

Although all entomopathogenic nematodes belong to the same group, they do not possess the same characteristics and therefore do not hold the same potential as biological control agents (Morton and Garcia-del-Pino 2009). Species characterization is therefore an important step during the selection of an appropriate isolate for the control of an insect pest in order to match the biological and ecological characteristics of the nematode isolate to that of the conditions associated with the target insect (Koppenhöfer and Kaya 1999; Shapiro-Ilan *et al.* 2002; Shapiro-Ilan *et al.* 2009; Morton *et al.* 2009).

The ideal nematode isolate would not only have to be virulent against codling moth, but would also have to be effective at lower temperatures and levels of water activity in cryptic habitats on trees and humidity, as can be expected in an orchard during application when targeting the suggested diapausing larval stage. The isolate would also have to possess an active searching behaviour in order to locate larvae residing in cryptic habitats on trees. These conditions have been proven to limit

nematode performance and therefore can be detrimental to the success of nematode isolates for the control of codling moth (Navaneethan *et al.* 2010).

Several isolates of *Steinernema feltiae* and *S. carpocapsae* have been shown to be effective control agents of codling moth (Lacey and Unruh 2005), based on the aforementioned requirements, such as being active at temperatures below 15°C and low levels of humidity (60 – 80% RH) (Navaneethan *et al.* 2010). In South Africa, however, neither of these two species have been isolated to date (Malan *et al.* 2006; De Waal 2008; Hatting *et al.* 2009) and current legislation prohibits the import of exotic species (Agricultural Pest Act 36 of 1947). All current work on the use of entomopathogenic nematodes for the control of codling moth in South Africa is therefore aimed at evaluating isolates which have been obtained from local soils. Most of these isolates have never been screened for virulence against codling moth and could possibly be effective control agents and therefore merit further investigation.

Thereupon, the objectives of the current study was to estimate the relative biocontrol potential of six South African entomopathogenic nematode isolates against diapausing codling moth larvae in various laboratory bioassays and field experiments regarding virulence, the effect of low temperatures, water activity and host seeking ability. The tested isolates were *Steinernema citrae* (141-C) Stokwe, Malan, Nguyen, Knoetze and Tiedt 2011, *Steinernema khoisanae* (J69 and SF87) Nguyen, Malan and Gozel 2006, *Steinernema yirgalemense* (157-C) Nguyen, Tesfamarian, Gozel, Gaugler and Adams 2005, an undescribed *Steinernema* sp. (J194) and *Heterorhabditis zealandica* (SF41) Poinar 1990.

## Materials and methods

### *Nematodes and insects*

The nematodes used in all the experiments were cultured in parallel at room temperature in final instar *Galleria mellonella* (L.). The emerging infective juveniles (IJs) were stored at 14°C for less than 2 weeks prior to use. Nematodes were quantified 1 hour before experimentation, using procedures

described by Kaya and Stock (1997). Nematodes used as test isolates for the various experiments were: *Steinernema citrae* (141-C), *S. khoisanae* (J69 and SF87), *S. yirgalemense (*157-C), *Steinernema* sp. (J194) and *Heterorhabditis zealandica* (SF41).

Codling moth eggs and diet were obtained from the codling moth rearing facility, Entomon Technologies (Pty) Ltd., Stellenbosch, Western Cape province, South Africa. From these eggs, larvae were reared on an artificial diet under diapausing conditions in growth chambers [photoperiod 10:14 (L:D), 25°C, 60% RH]. Fifth instar diapausing codling moth larvae were used for experimentation to avoid larvae pupating during the test period.

***Virulence***

A comparison of the virulence of *S. citrae* (141-C), *S. khoisanae* (J69 and SF87), *S. yirgalemense (*157-C), *Steinernema* sp. (J194) and *H. zealandica* (SF41) to fifth instar diapausing codling moth larvae was made. The rate of application and time of assessment were based on previous laboratory bioassays (De Waal 2008) and pilot trials. Multiwell plates (24 wells, volume 3.8 $cm^2$ per well, Flat bottom, Nunc™, Cat No. 144530) were used. Filter paper bioassay disks (13 mm in diameter) were placed in 10 wells in each plate. Based on the pre-determined dosage of $LD_{50}$ = 72 IJs/ml for *H. zealandica* (SF41) (De Waal 2008), an even lower dosage of 50 IJs/ 50 µl of filtered water per insect was chosen as the discriminating dosage for all tested isolates, with water only for the control treatments. After the inoculum had been applied onto the disks, one diapausing codling moth larva was added to each of the 10 wells. The lid of the bioassay plates was fitted with a 3 mm glass pane and kept secure with a rubber band to confine larvae to their wells. The plates were placed in plastic containers lined with moistened toweling and closed to maintain high humidity (> 95% RH). Containers, with plates, were placed in the dark in incubators at 25°C for 48 h, thereafter larvae were removed from trays and mortality was assessed. Four plates (each containing 10 larvae) were prepared for each of the six treatments and the experiment was repeated on four separate dates.

***Effect of low temperature on virulence***

Virulence of the same six nematode isolates to fifth instar diapausing codling moth larvae at average local winter day (17°C) and night (12°C) temperatures were compared. The same protocol as described in the virulence experiment was followed, except that the incubation temperature regime throughout the entire experiment was 17°C for 10 h to 12°C for 14 h in a 24 h cycle for 48 h, after which mortality was assessed. Four plates (each containing 10 larvae) were again prepared for each of the six treatments and the experiment was repeated on four separate dates.

***Effect of lower water activity on virulence***

In order to assess the performance of six different isolates against diapausing codling moth larvae at reduced humidity, the isolate was tested at different water activity levels ($a_w$ = 0.92; 0.94; 0.96; 1.00), which have been noted by Navaneethan *et al.* (2010) to be representative of the moisture levels on bark surfaces. The experiment was conducted using 3 cm diameter Petri dishes lined with filter paper. Different volumes of inoculum (50 IJs/larva suspended in water for treatments or only water in the case of untreated controls) were added to each dish to create the required $a_w$-values (eg. 12 µl inoculum was added to each dish to create an $a_w$-value of 0.94). The $a_w$-values were measured using the Decagon Pawkit water-activity-meter (Decagon Devices Inc., Pullman, WA, USA) at constant temperature (25°C). Thereafter, four diapausing codling moth larvae were added to each dish and dishes were covered with cling wrap and a lid to ensure an airtight seal. Dishes were incubated at 25°C to ensure nematode efficacy, for 48 h and thereupon larval mortality was assessed. Four replicates were prepared for both treatment and control dishes for each $a_w$-level tested for each of the six isolates, and the experiment was repeated on two separate dates.

***Host seeking ability***

The host seeking ability of the six isolates were evaluated. The experimental design was based on the host seeking experiment designed by Shapiro-Ilan *et al*. (2009), using codling moth larvae as test insects. Circular plastic containers (10 cm diameter, 4 cm height) were filled to approximately 2/3 depth with 2% agar. An inverted 0.5 ml microcentrifuge tube was inserted into the agar on opposite ends of each dish (4 mm from the edge of the plate), and a filter paper bioassay disk (13 mm in diameter) was placed in the centre of each dish on the surface of the agar. On treatment plates, one

diapausing codling moth larva was placed in one of the microcentrifuge tubes while the other tube was left empty (Image 1). In control dishes both tubes were left empty. To create a gradient of volatile host cues, the containers were left undisturbed for 2 h. Approximately 2000 IJs in 50 µl water were then applied to the bioassay disc of each dish. After 4 h, the number of IJs found in a 2 cm diameter circle around each tube was recorded. Host seeking ability (% attraction) was estimated by the number of IJs found in the circle with the host relative to the total number of IJs found in both the host and the empty tube's circle. In the control plates one side was randomly designated as the host side for calculation purposes. There were eight replicates per treatment and the trial was repeated on two separate dates.

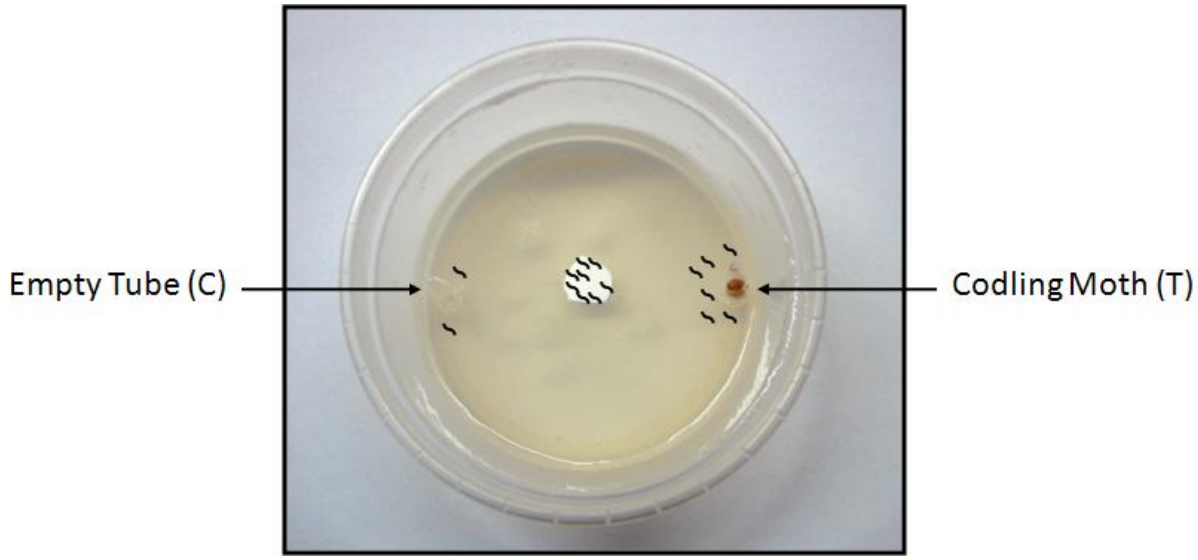


**Image 1**. A diagrammatic illustration of the experimental setup used in the host seeking ability experiment for treatment dishes, showing a filter paper disk onto which nematodes were inoculated and two microcentrifuge tubes, containing a codling moth larva (treatment) or an empty tube (control).

***Field performance***

The performance of *H. zealandica* (SF41), *Steinernema* sp. (J194) and *S. khoisanae* (J69) under field conditions was evaluated in a Forelle pear orchard on the experimental farm Welgevallen, located in Stellenbosch, Western Cape province, South Africa. A completely randomized block design was used for the experimental layout, with four rows, each containing eight treatment trees, with three buffer

trees between each treated tree and two buffer rows separating treatment rows. Each of the three nematode isolates was considered as a treatment, with the fourth treatment being a control treatment receiving water only. The experiment was repeated on two separate trial dates. Both trials were conducted just after sunrise during autumn 2010 (Trial 1: 22 April 2010 and Trial 2: 20 May 2010).

Three different insect containment methods (mesh cages, wooden planks and pear tree logs) were also compared during the two trials. Cylindrical mesh cages were based on the design used by Duncan *et al.* (2003). Each cage comprised a 40-mesh stainless steel cylinder (7 x 3 cm in diameter), fitted with polypropylene caps at each end. The day before each trial, cages were filled with pear bark chips and 20 diapausing codling moth larvae were added to each cage and allowed to spin into cocoons between the wood shavings at 25°C. Wooden planks (17.6 x 6.5 cm) were constructed from old fruit bin wood. Each plank was sawn in three parts and bolted back together again to facilitate easy removal of codling moth larvae from the planks at the termination of each experiment. Twenty holes (5 mm diameter, 2 cm apart) were drilled into the wood along the two grooves, adjoining the three pieces of wood. The day before each trial, each plank was placed in a 2 L plastic container and 20 diapausing codling moth larvae were added to each of these after which the lids were then closed and allowed to cocoon in the holes over a 24 h period at 25°C (De Waal *et al.* 2010). Pear tree logs were used as a third method of insect containment. Scaffold branches, obtained from a commercial Forelle pear orchard, were cut into 20 cm long logs and placed in a -20°C freezer for two days to eliminate other organisms before being placed at 30°C for another two days to dry. The day before each trial, 40 diapausing larvae were placed onto each branch and larvae were allowed to cocoon behind loose pieces of bark, or in pruning wounds on the branches, at 25°C.

**Image 2.** The different insect containment devices tested in the field experiment were (from left to right) wooden planks, mesh cages and pear tree logs.

On the day of each trial, containment structures were removed from containers and transported to the field where one of each of the insect containment structures was tied to each treatment tree close to the main stem (Image 2). All treatment applications were done using shoulder pump sprayers (Dal Degan, Italy). Trees were thoroughly pre-wetted (1 L per tree) 1 hour before treatment applications. Thereafter, nematodes at a concentration of 0.25 million IJs/tree (De Waal 2008) in 1 L water were applied evenly to the stem area containing the insect containment devices, approximately 1 m above ground level. Control plots were treated with 1 L of water only. To prevent nematodes from desiccating, trees were lightly misted every hour for six consecutive hours, taking care to minimize run-off. Infectivity and subsequent mortality was assessed thereafter.

***Statistical analyses***

All experiments were repeated on different test dates and the results combined for analysis. Abbott's formula was used to correct the data for control mortality (Abbott 1925). All statistical analyses were performed using Statistica 9.0 software (Statsoft Inc. 2009). ANOVA was used to compare the

different treatments, followed by Bonferroni multiple comparisons. If residuals were not normally distributed, Bootstrap multiple comparisons were used (Efron and Tibshirani 1993). To statistically evaluate the water activity experiment, a Probit analysis was conducted using Polo PC (LeOra Software 1987). The $aw_{50}$ values were estimated with their 95% fiducial limits. All values throughout the text are given with corresponding standard errors or fiducial limits.

## Results

### *Virulence*

In general, the data suggested codling moth larvae to be highly susceptible to all nematode isolates tested. There were, however, significant differences amongst these isolates (F = 4.703; df = 5,90; P = 0.0007). The lowest level of mortality was recorded for *S. citrae* (141-C) at 84.74 ± 2.82% as opposed to the remaining treatments, where the levels of mortality ranged from 87 - 100% (Figure 1).

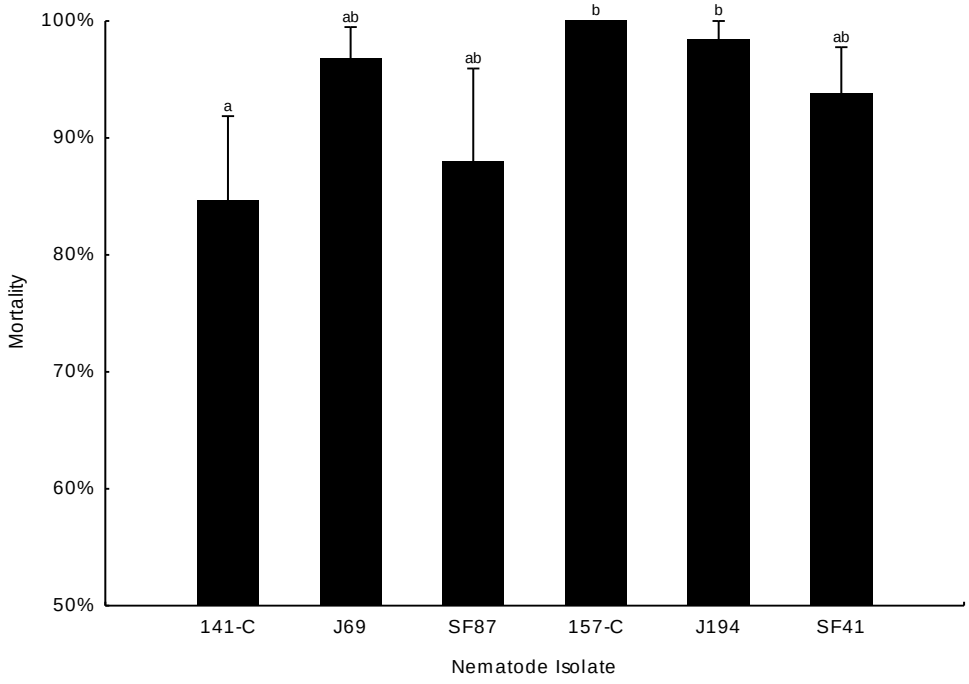


**Figure 1.** Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after exposure to *Steinernema citrae* (141-C), *S. khoisanae* (J69 and SF87), *S. yirgalemense*

*(*157-C), *Steinernema* sp. (J194) and *Heterorhabditis zealandica* (SF41) at a concentration of 50 IJs/insect. Different lettering above vertical bars indicates significant differences (one-way ANOVA; F = 4.703; df = 5,90; P = 0.0007).

***Effect of low temperature on virulence***

Mortality recorded for codling moth larvae at low temperatures (10 h 17°C; 14 h 12°C) was below ≤ 3% for six nematode isolates tested, with no significant differences in mortality levels amongst isolates (F = 1.17; df = 5,90; P = 0.33), as opposed to the high levels of mortality recorded at 25°C as illustrated in the virulence experiment.

***Effect of lower water activity on virulence***

All the water activity ($a_w$) levels tested caused some degree of larval mortality for all isolates tested, with the lowest levels of mortality at $a_w$=0.92 and maximum mortality values at $a_w$=1.00. There was a positive relationship between the increasing water activity level and degree of larval mortality. The average $a_{w50}$-values for all isolates tested was 0.94 (0.93 - 0.95), except *S. khoisanae* (SF87) 0.97 (0.97 - 0.98).

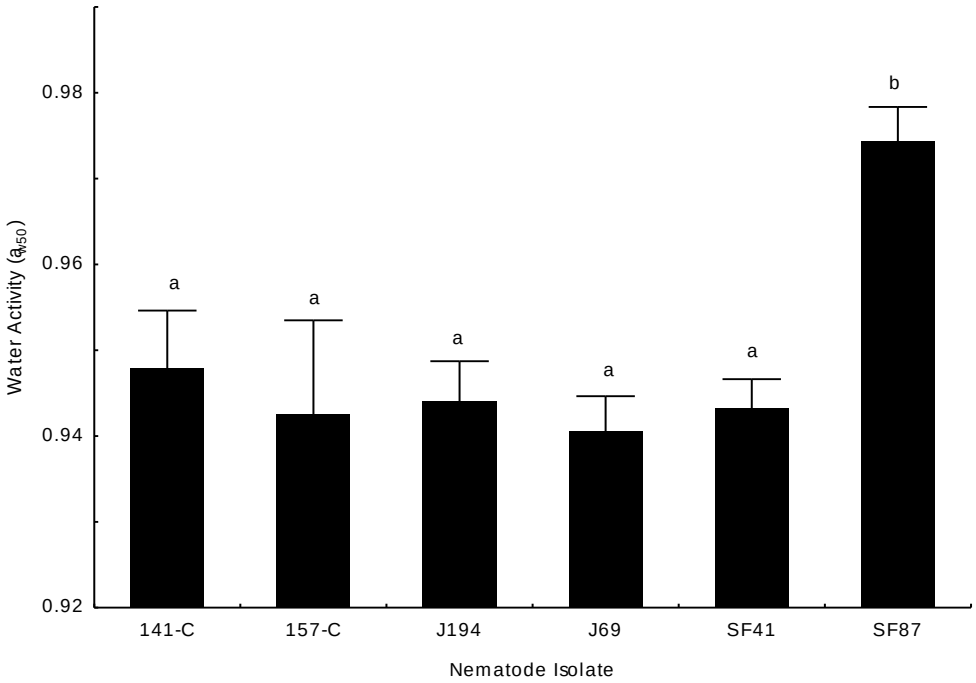

**Figure 2.** Mean water activity level and corresponding confidence intervals causing 50% diapausing codling moth larval mortality ($a_{w50}$) after exposure to *Steinernema citrae* (141-C), *S. khoisanae* (J69 and SF87), *S. yirgalemense (*157-C), *Steinernema* sp. (J194) and *Heterorhabditis zealandica* (SF41) at a concentration of 50 IJs/insect for 48 h.

***Host seeking ability***

None of the nematode species exhibited positive attraction (< 50%) to the host, as no significant difference could be detected between treatment and control plates (F = 1.88; df = 5,180; P = 0.09), except for *H. zealandica* (SF41). Based on the result that the percentage of nematodes that moved toward codling moth cues did not differ significantly for any of the isolates, except *H. zealandica* (SF41) (F = 6.57; df = 5, 180; P < 0.001), no significant difference in host-seeking ability was detected among the remaining nematode species (Figure 3).

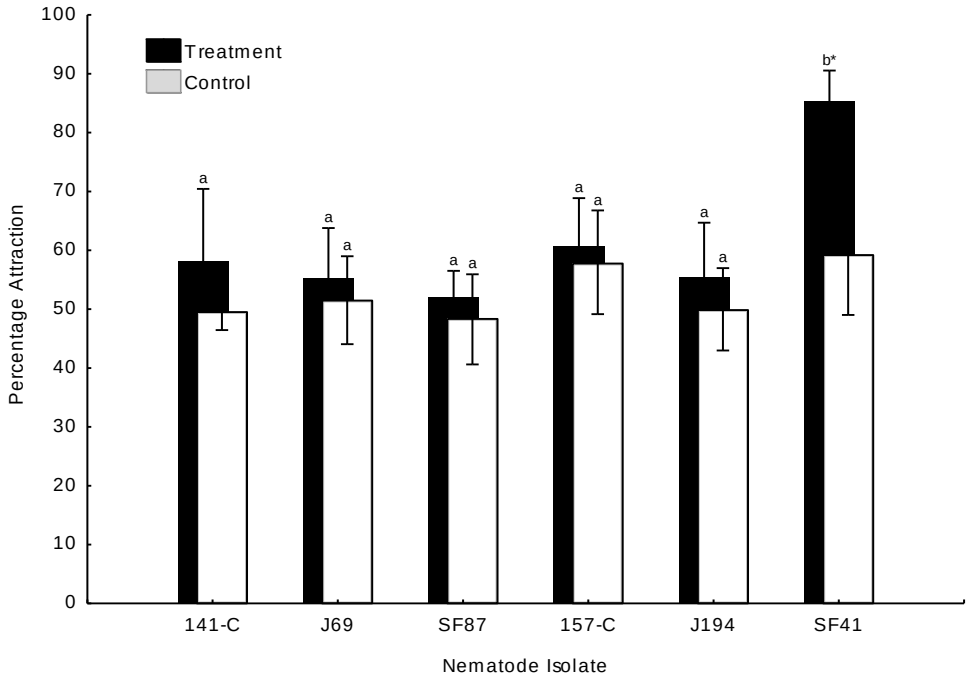


**Figure 3.** Mean percentage of infective juveniles (95% Confidence Interval) that moved toward codling moth host cues (Treatment), or toward a randomly selected side of a dish that did not contain host cues (Control) as recorded for *Steinernema citrae* (141-C), *S. khoisanae* (J69 and SF87), *S. yirgalemense* (157-C), *Steinernema* sp. (J194) and *Heterorhabditis zealandica* (SF41). Different lettering above vertical bars indicates significant differences between treatment and control dishes

within each isolate (factorial ANOVA; F = 1.88; df = 5,180; P = 0.09) and asterisks indicate statistical differences amongst nematode species (factorial ANOVA; F = 6.57; df = 5,180; P < 0.001).

***Field performance***

The average levels of mortality for all tested nematode isolates ranged between 52-70% for both trials under field conditions. There were minimal climatic differences between the two trials, as summarized in Table 1.

**Table 1.** Climatic data recorded during two trial periods (Trial 1: 23/04/2010 and Trial 2: 21/05/2010) in the orchard.

| | Trial 1 | Trial 2 |
|---|---|---|
| Temperature avg. (min-max) °C | 20 (14 – 27)°C | 24 (16 – 26)°C |
| RH avg. (min-max) % RH | 48 (32 – 99) % RH | 33 (13 – 72) % RH |
| Rainfall total (mm) | 0.6 mm | 0.2 mm |
| Wind avg. (m/s) | 6.8 m/s | 2.8 m/s |

The total percentage mortality recorded for both trials irrespective of monitoring method was calculated to further highlight the differences among the different treatments, using a one-way ANOVA. Significant differences could subsequently be detected among treatments (F = 20.37; df = 3,60; P < 0.001). The highest level of control was obtained for *Steinernema* sp. (J194) (70.27 ± 4.05%), followed by *H. zealandica* (SF41) (59.25 ± 4.05%) and *S. khoisanae* (J69) (52.91 ± 4.05%) and (Figure 4).

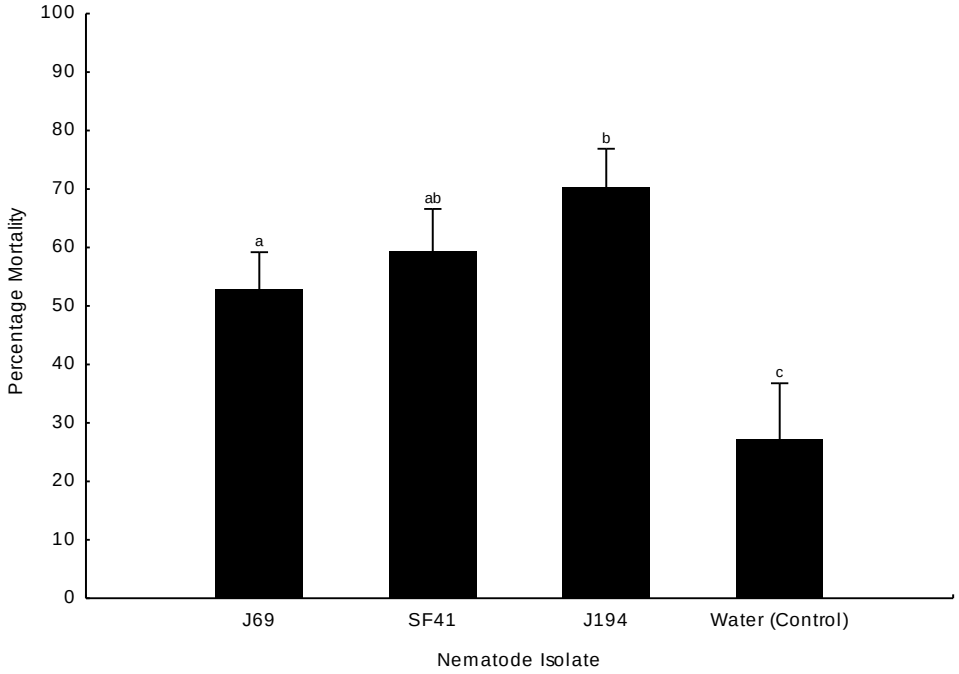


**Figure 4.** Mean percentage mortality (95 % Confidence Interval) recorded for diapausing codling moth larvae after exposure to *Steinernema khoisanae* (J69), *Heterorhabditis zealandica* (SF41), *Steinernema* sp. (J194) and water as a control treatment during field experiments. Different lettering above vertical bars indicate significant differences (one-way ANOVA; $F = 20.37$; $df = 3,60$; $P < 0.001$).

Mortality levels recorded using different insect containment methods (mesh cages, wooden planks and pear tree logs) for host deployment during field experimentation, was analyzed using a repeated measures ANOVA (using containment method as the repeated factor). Significant differences were found between the different methods ($F = 134.28$; $df = 2,126$; $P < 0.001$). The highest level of mortality was obtained when using mesh cages (71.62 ± 2.64%), as opposed to the lower levels of mortality recorded when using wooden planks (55.76 ± 3.13%) and pear tree logs (29.76 ± 3.13%) (Figure 5).

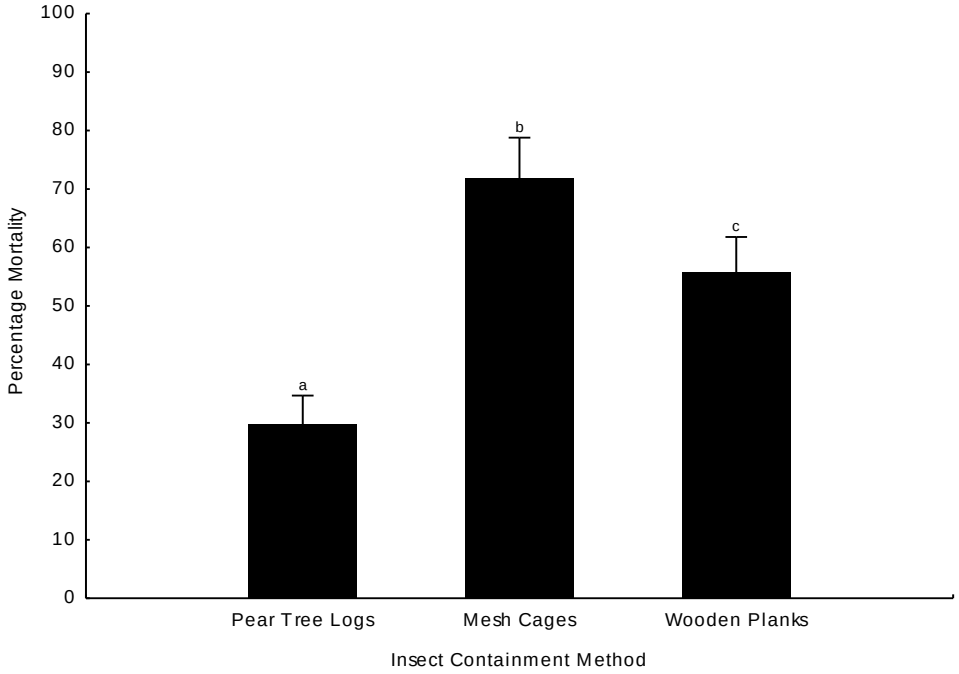


**Figure 5.** Mean percentage mortality (95 % Confidence Interval) recorded for diapausing codling moth larvae using various insect containment methods (pear tree logs, mesh cages and wooden planks) for host deployment after exposure to *Steinernema khoisanae* (J69), *Heterorhabditis zealandica* (SF41) and *Steinernema* sp. (J194). Different lettering above vertical bars indicate significant differences (repeated measures (ANOVA; F = 134.28; df = 2,126; P < 0.001).

Predictive relationships between the percentage mortality obtained for pear tree logs (Y) vs. the percentage mortality obtained for mesh cages and wooden planks (X), was determined using linear-regression analyses. Regression analyses were done separately for each nematode isolate, as significant differences were previously detected in the field performance of the different nematode isolates (Figure 4). The resulting regression equation and percentage variation explained by the regression for each isolate is summarized in Table 2. For the three nematode isolates tested, the percentage variation was always greater for the mortality recorded for the planks as opposed to the cages.

**Table 2.** Regression equations and corresponding values for *Steinernema khoisanae* (J69), *Heterorhabditis zealandica* (SF41) and *Steinernema* sp. (J194) to estimate the percentage mortality which can be expected on pear tree logs (Y), using percentage mortality obtained in mesh cages or wooden planks as predictors (X).

| Nematode Isolate | X | Regression Equation | % Variation | P-value |
|---|---|---|---|---|
| *Steinernema khoisanae* (J69) | % Mortality(planks) | Y = -6.69 + 0.64 X | 54.97% | 0.001 |
| | % Mortality (cages) | Y = -24.00 + 0.69 X | 22.20% | 0.066 |
| *Heterorhabditis zealandica* (SF41) | % Mortality(planks) | Y = -15.68 + 0.81 X | 88.18% | <0.001 |
| | % Mortality (cages) | Y = -68.97 + 1.02 X | 29.28% | 0.030 |
| *Steinernema* sp. J194 | % Mortality(planks) | Y = -66.80 + 1.44 X | 79.86% | <0.001 |
| | % Mortality (cages) | Y = -10.65 + 0.68 X | 37.88% | 0.011 |

## Discussion

This study evaluated the biocontrol potential of five *Steinernema* isolates and one *Heterorhabditis* isolate, regarding certain biological and ecological characteristics fundamental to the success of controlling codling moth, in laboratory bioassays and field trials.

Virulence is the foremost important prerequisite for a nematode isolate to be considered as having biocontrol potential against an insect pest. The high levels of mortality (> 84%) recorded in the virulence experiment, evaluating all isolates against diapausing codling moth larvae, confirmed that all nematode isolates tested adhered to this requirement and could consequently be used in further trials throughout the rest of the study. Our results were consistent with previous work showing codling moth to be very susceptible to various isolates of entomopathogenic nematodes (Kaya *et al.* 1984; Lacey *et al.* 1998; Lacey and Chauvin 1999; Vega *et al.* 2000; Unruh *et al.* 2001; Cossentine *et al.* 2002; Lacey *et al.* 2005; Lacey *et al.* 2006a; Lacey *et al.* 2006b; Lacey *et al.* 2007; De Waal 2008; Malan *et al.* 2009).

The next step in evaluating the biocontrol potential of the test isolates against codling moth was to investigate whether they would be pathogenic to larvae under conditions as can be expected in the field. The proposed time to apply these nematodes is in late summer, through winter till early spring (Lacey *et al.* 1998), which implies that the ideal nematode isolate would have to be effective against codling moth larvae at moderately low temperatures. In South Africa average local winter temperature

is approximately 17°C during the day and 12°C during the night. The potential of the test isolates to infect codling moth larvae at these temperatures was therefore evaluated. Low levels of mortality (≤ 3%) were obtained for all isolates, indicating that it would be better to apply the nematodes during the autumn or spring when higher temperatures can be expected. These results are consistent with previous work conducted by Lacey et al. (1998), where temperatures below 15°C were also shown to limit the efficacy of nematodes to infect codling moth larvae. It should however be pointed out that on some days during the winter, temperature will still be within the optimum temperature range (20 - 25°C) for nematode activity (Koppenhöfer 2007) for a number of hours, which could ensure successful infection.

As previously mentioned, diapausing codling moth larvae are known to occur in cryptic habitats such as cracks and crevices on trees, pruning wounds and underneath and between loose pieces of bark. The ideal nematode isolate for codling moth management would therefore have to be effective above-ground, and able to withstand lower levels of humidity (Lacey *et al.* 1998; Koppenhöfer 2007). Hypothetically, this would be detrimental to nematode survival, as nematodes require a thin water film to maintain activity and ensure host infection. It has thus been proposed that moisture is one of the more important factors when evaluating the selection of an effective nematode isolate for the control of diapausing codling moth larvae.

Lacey and Unruh (1998) showed that nematodes were only active at maximum humidity (> 95% RH). However, ambient humidity cannot be used as the only indication of nematode performance. Conditions in the field in the macro-environment (surrounding orchard humidity) differ from the micro-environment (cryptic habitat where larvae are residing eg. underneath loose pieces of bark on the tree) (Navaneethan *et al.* 2010). As previously mentioned, water activity ($a_w$-value) has been noted to give an indication of the available water on the bark surface of trees and therefore the effectiveness of nematodes in this micro-environment (Koppenhöfer 2007). Navaneethan *et al.* (2010) documented the first investigation of the influence of water activity on nematode efficacy, using *S. feltiae*. The current study elaborated on this concept, showing that all isolates tested were not necessarily dependent on a water film ($a_w = 1$) to infest codling moth larvae, as larvacidal activity was still recorded down to $a_w = 0.92$. Bark from living trees have been noted to have an $a_w$-value of 0.965 (Navaneethan *et al.* 2010), which would therefore support nematode activity for all isolates, except *S. khoisanae* (SF 87). The water activity level which caused 50% mortality for all isolates was established to be

approximately 0.94, which is similar to results obtained by Navaneethan *et al.* (2010), where the $a_{w50}$-value was 0.95 for cocooned larvae.

Another important characteristic to consider for nematode isolate selection for codling moth control is host seeking-ability (Griffin *et al.* 2005), as the nematodes need to move into the cryptic areas where the larvae are residing on the tree to infect them. Dispersal behaviour and capabilities of EPNs vary among species and isolates (Lewis 2002), and the host-seeking experiment was therefore undertaken to determine differences amongst the test isolates. Positive attraction of the tested isolates to codling moth larvae in the host-seeking experiment could only be confirmed for *H. zealandica* (SF41), as equal distribution was observed for the other isolates (Figure 3). Lewis (2002) proposed that a nematode's response to host cues can be linked to foraging behaviour, and that ambushing nematodes would therefore not respond as strongly to host cues as would cruise-foraging nematodes. This hypothesis could explain the good results obtained for *H. zealandica* (SF41), as *Heterorhabditis* generally utilize a cruising strategy to locate their hosts (Susurluk *et al.* 2003). This could also be motivated further by previous research showing that *H. zealandica* has a high innate dispersal rate in soil (Koppenhöfer and Fuzy 2008) and in different mulch substrates (De Waal *et al.* 2011). It should, however, be avoided to draw direct conclusions on foraging behaviour and movement ability for the remaining isolates based on the current experimental design, as it has also been noted that substrate contributes to behaviour (Lewis 2002). Agar has been shown to limit nictation and jumping in some species (Lewis 1993, Lewis *et al.* 2006), and it would therefore be advisable to rather use a more natural substrate such as bark, to get a true indication of foraging behaviour and host seeking ability. However, the results obtained were still included in the ranking.

Laboratory conditions are not necessarily representative of field performance and the final experiment in the current study was to evaluate the potential of *H. zealandica* (SF 41), which has previously been shown to be effective against codling moth (De Waal *et al.* 2010), together with *S. yirgalemense* (157-C) and the undescribed *Steinernema* sp. (J194), which have never been tested in a field experiment.

Entomopathogenic nematodes are known to be sensitive to low levels of humidity and cold temperature, which are the most important climatic conditions in the case of codling moth (Lacey and Unruh 2005; De Waal 2008). Moderate temperatures (≥ 15°C and ≤ 30°C), as recorded during both trials (Table 1), have proved to be favourable to nematode survival. That would subsequently have

positively influenced the level of mortality obtained in both trials (Koppenhöfer 2007), as opposed to the low levels of humidity recorded (Table 1). Theoretically, low levels of humidity should have been detrimental to nematode performance as noted in literature (Wright *et al.* 2005). However, this was not evident from the results of the field trials, as satisfactory levels of larval mortality (> 50%) were still obtained. This would indicate that the humidity and possibly even the temperature recorded in the field differed from the actual level of humidity on the bark itself, and could therefore be regarded as two separate climatic environments. This corroborates with Navaneethan *et al.* (2010)'s theory that to obtain climatic data which is truly reflective of nematode performance, separate recordings in both the macro- and micro-environments have to be taken.

Field experiments showed deviating but satisfactory levels of mortality for *H. zealandica* (SF41), *S. khoisanae* (J69) and *Steinernema* sp. (Figure 4) and it can consequently be deduced that all these isolates were effective against diapausing codling moth under optimum field conditions.

As part of an investigation of the effect of using entomopathogenic nematodes for the control of codling moth, various insect containment methods have been used, including cardboard strips (Lacey *et al.* 1998), mesh cages (De Waal *et al.* 2011), wooden planks (De Waal *et al.* 2010), apple wood logs (Lacey *et al.* 1998) and cardboard tree bands (Kaya *et al.* 1984). Preparation of some of these methods has been noted to be time consuming and labour-intensive and codling moth larvae have also been reported to escape from some of these devices (De Waal *et al.* 2011). Retrieving larvae post-treatment in this specific study from the pear tree logs was very difficult, as they either spun their cocoons underneath pieces of bark (which then needed to be peeled from the log) or burrowed themselves into the wood. There is also the concern that these host deployment methods are not representative of the natural situation. Although being sufficient for inter-experimental analyses and conclusions, the higher levels of control obtained using these containment methods would not be representative of the actual level of control which would be obtained in the field for larvae residing in cryptic habitats, such as pruning wounds or underneath pieces of bark on the trees.

This speculation was confirmed in the current study, as notably different levels of mortality were obtained between the different insect containment methods tested (Figure 5). The higher levels of mortality recorded for the mesh cages and wooden planks could be ascribed to the nematodes being able to reach the larvae more easily, as opposed to on tree logs, where larvae would reside

underneath loose pieces of bark, or in pruning wounds, which would make it more difficult for the nematodes to get in contact with the larvae.

Tree logs would therefore be the most representative host deployment method to use in field trials, however, as previously mentioned it was very difficult to retrieve the larvae from the logs at the termination of the trial. For this reason, and the fact that there was a direct relationship between the level of control obtained on the tree logs and either the mesh cages or wooden planks, regression equations (Table 2) were determined, which would enable future field experiments to be conducted with either planks or mesh cages, whilst being able to determine what the level of control would have been on tree logs, as a representative of the wild type situation. Between planks and mesh cages, lesser variation were observed for planks for all isolates (Figure 5), indicating that planks would be the best containment method to be used in experimentation.

In conclusion, the study illustrated that all isolates tested showed some degree of biological control potential against diapausing codling moth larvae, regarding larvacidal susceptibility and field performance. However, none of the experiments clearly showed any of the isolates to be significantly more effective against codling moth. The study also highlighted the detrimental effect that environmental factors (such as low temperatures and moisture levels) could have on the success of an application. Future research should therefore be directed at overcoming some of these limiting factors for improved control of the tested isolates for the management of codling moth in temperate regions.

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# CHAPTER 5

## A superabsorbent polymer formulation for improved efficacy of *Heterorhabditis zealandica* (Rhabditida: Heterorhabditidae) for the control of codling moth larvae, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae)


### Abstract

Formulation of nematodes aimed at above-ground applications, may assist in maintaining adequate moisture levels on the application substratum required for nematode survival and subsequent efficacy. Here we report the effects of using the entomopathogenic nematode, *Heterorhabditis zealandica*, together with a superabsorbent polymer formulation, Zeba®, to control diapausing codling moth, *Cydia pomonella* larvae in cryptic habitats on trees. Water activity ($a_w$-value) on bark was considered to be an indication of moisture levels on trees in cryptic habitats where codling moth larvae are known to occur, thereby influencing nematode efficacy. *Heterorhabditis zealandica* was only able to infect codling moth larvae at $a_w \geq 0.92$, with $a_{w50} = 0.94$ and $a_{w90} = 0.96$. Laboratory experiments in which nematode concentration was investigated, indicated a positive linear relationship between nematode concentration and the level of control obtained, with the highest level of mortality recorded at 80 IJs/larva. A minimum exposure time of 4 h was required to ensure nematode infectivity and subsequent efficacy. The use of the Zeba®, improved the level of control obtained at 60% and 80% RH in the laboratory, and also enhanced the survival and infection-ability of nematodes in the field. The study illustrated that Zeba® assisted in maintaining adequate moisture levels on the application substratum, as required for nematode survival and subsequent efficacy.


### Introduction

Codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) is a devastating pest of apples and pears throughout temperate regions of the world (Barnes 1991). Adult moths emerge from pupae in late spring and lay their eggs individually on the fruit and adjacent leaves. Neonate larvae hatch from these eggs and move towards the fruit, where they penetrate into the fruit to feed, creating a visible frass-filled tunnel. This action and its resultant appearance reduce the market value of the crop (Welter 2008). Final instar larvae then exit from the fruit in search of a cryptic habitat (mainly on trees), where they either cocoon and pupate, or overwinter as diapausing larvae (Blomefield 2003). Depending on temperature, codling moth may go through one to four generations per growing season (Barnes 1991).

Codling moth control previously involved the extensive use of broad-spectrum insecticides (Giliomee and Riedl 1998). However, concerns over human safety, environmental impact, widespread dispersal of resistant populations of codling moth and the sustainability of synthetic pesticides in agroecosystems, has encouraged the development and use of alternative pest management technologies (Blomefield 2003). These include biological control agents such as entomopathogenic nematodes (Kaya *et al.* 1984; Lacey and Unruh 1998).

Entomopathogenic nematodes belong to the families Steinernematidae and Heterorhabditidae and are able to kill insects with the aid of a mutualistically-associated bacterium (*Xenorhabdus* spp. in heterorhabditids and *Photorhabdus* spp. in steinernematids (Boemare 2002). The nematodes generally complete two to three generations within the host, after which a non-feeding and developmental arrested third-stage dauer juvenile emerges to seek new hosts. This stage is generally referred to as the infective juvenile (IJ) stage in which nematodes enter host insects through natural openings such as the mouth, anus, spiracles, and occasionally through the cuticle, whereupon the associated bacteria are released inside the insect, normally killing the insect within 48 h (Poinar 1990).

Although entomopathogenic nematodes are generally pathogenic to a wide variety of insect pests (Poinar 1979), successful commercialization and the use of the nematodes for biological control on a commercial basis have been limited to relatively few target insects (Shapiro-Ilan *et al.* 2002). Codling moth has not only been proven to be susceptible to entomopathogenic nematodes in several research experiments (Kaya *et al.* 1984; Lacey and Unruh 1998; Lacey and Chauvin 1999; Vega *et al.* 2000; Unruh and Lacey 2001; Cossentine *et al.* 2002; Navaneethan *et al.* 2010), but it is also one of the few

examples where nematodes are used on a commercial basis to help control this insect pest in integrated pest management systems.

The developmental stage of codling moth best suited for control with nematodes is the diapausing larval stage, occurring between late summer and early spring in cryptic habitats such as underneath loose pieces of bark or in pruning wounds on trees (Lacey and Unruh 1998; Navaneethan *et al.* 2010). Eliminating this stage would protect fruit from damage in the following growing season (Lacey and Unruh 1998; Lacey *et al.* 2007).

Due to the above-ground location of diapausing codling moth larvae and the suggested seasonal time of application during autumn and winter (Glazer 2002; Navaneethan *et al.* 2010; Lacey *et al.* 2010), low temperatures and low levels of relative humidity (RH) have proved to be the most limiting factors in the use of these nematodes for the control of codling moth. In colder fruit production regions, cold-adapted species, such as *Steinernema feltiae* could be applied, since it has been proven to be effective at temperatures below 15°C (Grewal *et al.* 1996; Lacey and Unruh 1998). In more temperate regions, however, low temperatures are not such a problem. It is the issue of low levels of RH together with heat rather, that causes nematodes to desiccate even quicker than would normally be the case in regions with colder climates. Genetically improved nematode isolates can partially help to overcome this problem, manipulation of the habitat where the nematodes will be applied can however offer a practical solution (Webster 1973). Improved nematode-formulation through the addition of certain hydroscopic additives has proved to partially prevent nematodes from rapid desiccation (Glazer 2002; Navaneethan *et al.* 2010; Lacey *et al.* 2010).

The overall objective of the present study was to contribute to the aforementioned research field by an evaluation of the superabsorbent polymer formulation known as Zeba® [starch-g-poly(2-propenamide-co-2-propenoic acid) potassium salt, Tongaat Hulett Starch] for the improved performance of the entomopathogenic nematode *Heterorhabditis zealandica* Poinar 1990 (SF 41) for the control of codling moth. The specific aims of the study were (1) to determine the required water activity level ($a_w$-value), (2) optimal concentration, (3) exposure time required by the nematodes to cause satisfactory levels of larval mortality at different levels of humidity and (4) to further investigate whether the addition of the starch-based formulation would improve the efficacy of the nematodes in semi-natural conditions in the laboratory and (5) during field application.

## Materials and methods

### *Nematodes and insects*

Infective juveniles (IJs) of *H. zealandica* (Malan *et al.* 2006) were produced in *Galleria mellonella* (L.) larvae at room temperature. Harvested nematodes were stored horizontally in 150 ml filtered water in vented 500 ml culture flasks at 14°C, and shaken weekly for aeration. One hour before commencing each experiment, IJ concentrations for all trials were quantified in the laboratory, using procedures described by Kaya and Stock (1997). Codling moth eggs and diet were obtained from the codling moth rearing facility, Entomon Technologies (Pty) Ltd., located in Stellenbosch, Western Cape, South Africa. From these eggs, larvae were reared on an artificial diet under diapausing conditions in growth chambers [photoperiod 10:14 (L:D), 25°C, 60% RH]. Fifth instar diapausing codling moth larvae were used for all experiments.

### *Bioassay protocol*

To simulate semi-natural conditions, all experiments, except the water activity trial, were conducted with pear tree trunks, 10 cm in length, with a circumference of approximately 17 cm (Image 1). Trunks were obtained from a commercial pear orchard on the farm Timberley, located in the Stellenbosch district, Western Cape, South Africa. Trunks were kept at 45°C for two days to dry and eliminate other organisms. Six holes (1 cm deep, 0.5 cm wide) were drilled into each trunk and a diapausing codling moth larva was transferred to each hole. Larvae were allowed to spin up in cocoons inside the holes over a 24 h period prior to each experiment. For nematode application during the laboratory bioassays, an airbrush sprayer was used at 2 atm pressure. Nematodes were applied at a concentration of 80 IJs/larva in 5 ml of water, except in the concentration experiment, where nematodes were applied at 0, 5, 10, 20, 40 and 80 IJs/larva. Controls were treated similarly, but with water only. To create the desired level of humidity, trunks were placed in sealed plastic containers,

containing saturated salt solutions specific to each experiment. Containers were then incubated in climate chambers for four days at the start of a continuous temperature cycle of 10 h at 22°C, followed by 14 h at 11°C, to simulate average autumn temperatures in temperate regions. Temperature and humidity levels were monitored by Hobo® H8 Pro Series data loggers, which were placed inside the climate chambers. After four days of incubation, larvae were removed from the trunks and mortality assessed by prodding insects gently for movement.

**Image 1.** Pear tree trunks (10 cm length, 17 cm circumference) used for insect containment.

***Effect of water activity levels on codling moth mortality***

In order to assess the performance of *H. zealandica* against diapausing codling moth larvae at reduced humidity, the isolate was tested at different water activity ($a_w$) levels. Adjusting and maintaining different $a_w$-values on pear tree trunks were impractical, and the experiment was therefore conducted in 3 cm diameter Petri dishes lined with filter paper. Different volumes of water containing 300 IJs or only water in the case of controls, were added to each dish to create the required $a_w$-values. The $a_w$-values were measured using the Decagon Pawkit water-activity-meter (Decagon Devices Inc., Pullman, WA, USA) at a constant temperature (25°C). Thereafter, five diapausing codling moth larvae were added to each dish, covered with cling wrap and a lid to ensure an airtight seal. Dishes were incubated at the optimum infectivity temperature of 25°C for 48 h, whereafter larval mortality was assessed. Five replicates were prepared for both treatment and control dishes for each $a_w$-level tested, and the experiment was repeated on two separate dates.

***Influence of different nematode concentrations and relative humidity on codling moth mortality***

Using the previously described standard bioassay protocol, the effect of different nematode concentrations and levels of humidity on mortality was assessed. Saturated salt solutions in closed plastic containers were used to achieve 60% (glycerol), 80% ($KNO_3$) and 100% RH in containers lined with moistened tissue paper. Five trunks (each containing 6 diapausing codling moth larvae) were prepared for each of the different nematode concentrations (0, 5, 10, 20, 40 and 80 IJs/larva in 5 ml of water) at each of the different levels of humidity (60, 80 and 100% RH), and the experiment was repeated on two separate dates.

***Influence of exposure time on codling moth mortality***

To ensure infectivity under ideal conditions, the influence of exposure time on larvacidal activity was assessed using the standard bioassay protocol to determine how long trees should be kept moist. Ten trunks (each containing six diapausing codling moth larvae) were prepared for each of the time-intervals tested. Nematodes were applied to trunks at a concentration of 80 IJs/larvae in 5 ml of water and incubated at 100% RH in sealed plastic containers lined with moistened filter paper. An equal number of trunks were also prepared for control treatments, where the same volume of water only was applied to each trunk. After treatment and the subsequent incubation-period, larvae were removed from trunks at 30, 60, 180, 240 and 480 min intervals, rinsed with filtered tap water to remove surface nematodes and placed in Petri dishes lined with moistened filter paper to allow nematode development for a further three days, after which mortality was assessed. The experiment was repeated on two separate days.

***Effect of a Zeba® formulation in tree trunk laboratory bioassay***

To test the superabsorbent polymer known as Zeba® [starch-g-poly(2-propenamide-co-2-propenoic acid) potassium salt, Tongaat Hulett Starch], together with the nematodes at a concentration of 3 g Zeba®/L water, tests were conducted using pear tree trunks and the standard bioassay procedure was followed. The only alteration being that the logs were pre-wetted to an $a_w$-value of 0.96 to

simulate the general bark surface moisture of a live tree (Navaneethan *et al.* 2010). As described in the concentration experiment, saturated salt solutions in closed plastic containers were again used to achieve 60% and 80 % RH for incubation. Ten trunks, each containing six diapausing codling moth larvae, were prepared for each of the two treatments, which included (1) Zeba® + nematodes and (2) nematodes only. For control treatments, an additional ten trunks which received water only, were prepared for each of the treatments. Nematodes were again applied at a concentration of 80 IJ/larva in 5 ml of water. The trial was repeated on two separate dates and at two different levels of humidity (60% and 80% RH). Water activity ($a_w$-value) was also measured for each of the treatments at the start and again at the end of the trial period.

***Field application***

The effect of formulation on the efficacy of *H. zealandica* against diapausing codling moth larvae under field conditions was evaluated in a Forelle pear orchard on the experimental farm, Welgevallen, in the Stellenbosch district, Western Cape, South Africa. The field experiment was conducted mid-morning during winter on 14 June 2011. A completely randomized block design, with four rows, each containing eight treatment trees, with three buffer trees between each treated tree and two buffer rows separating treatment rows was used for the experimental layout. As previously described in the standard bioassay protocol, pear tree trunks, each containing 10 diapausing moth larvae were used for insect containment in the field experiment. Treatments were: (1) nematodes, (2) nematodes + Zeba®, (3) nematodes + Zeba® + Nu-Film-P® (poly-1-p-menthene, spreader/sticker, Hygrotech) and (4) water, as a control treatment. Nematodes were applied at a concentration of 50 IJs/cm$^2$, Zeba® at 3 g/L water and Nu-film-P® at 0.6 ml/L water. Treatment formulations were prepared on the day of the trial. Three trunks, containing codling moth larvae, were fastened to each of the 32 treatment trees onto the scaffold branches 1 m above ground on the day of the trial (Image 2). Treatment trees and trunks were thoroughly pre-wetted (1 L water per tree) 1 h before treatment applications. Thereafter, 20 ml of treatment suspension was applied directly onto each trunk. All treatment applications were done using calibrated hand-held spray applicators. After 24 h, trunks were removed from trees and taken back to the laboratory. Larvae were then removed from the trunks, washed to remove surface nematodes, placed in Petri dishes lined with moistened filter paper (Whatman No. 1) and incubated for a further 48 h at 25°C, whereupon larval mortality and nematode infection were assessed.

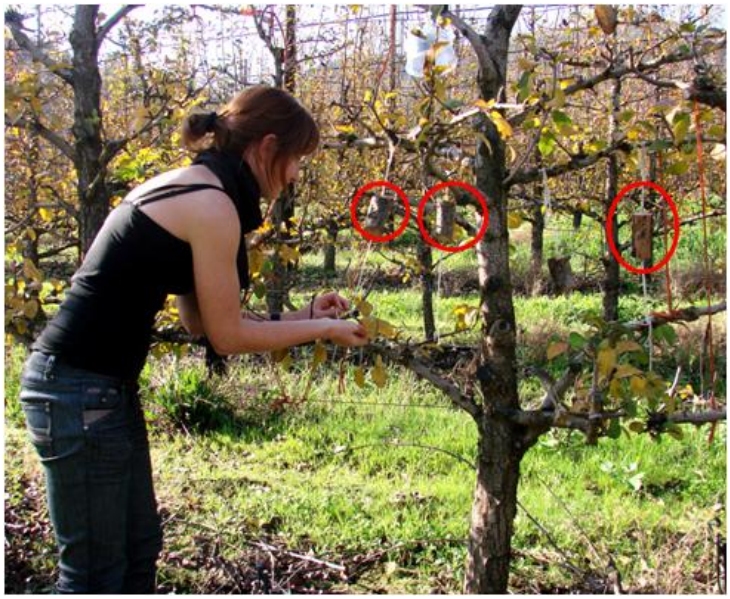

**Image 2.** Pear tree trunks were fastened to treatment trees on the morning of the field experiment.

To evaluate the longevity of nematodes on trunks during the field trial period, four pieces of bark (± 2 $cm^2$) were removed manually from treatment trunks every hour for the first eight hours and rinsed in 50 ml filtered water. The resulting water sample was then transferred to 50 ml glass cylinders and nematodes were allowed to settle at the bottom. A 5 ml volume of the resulting concentrate was examined under the microscope and the percentage of IJ survival was determined by movement response when probed with a dissecting needle (Kaya and Stock 1997). Pieces of bark were also removed from treatment trunks hourly and placed in Petri dishes, to which codling moth larvae were added, to determine infectivity at each of the different hourly intervals, for each of the nematode treatments.

Throughout the trial period, weather data were downloaded from the Helderberg Weather Station in Stellenbosch and Hobo® H8 Pro Series data loggers were placed in the middle of each treatment row on a scaffold branch of a tree to monitor temperature and humidity in the orchard environment.

***Statistical analyses***

All laboratory experiments were repeated on different test dates and the results combined for analysis. Abbott's formula was used to correct the data for natural mortality (Abbott 1925). All statistical analyses were performed using Statistica 9.0 software (Statsoft Inc. 2009). ANOVA was used to

compare the different treatments, followed by Bonferroni multiple comparisons. When residuals were not normally distributed, Bootstrap multiple comparisons were used (Efron and Tibshirani 1993). To statistically evaluate the water activity experiment, a Probit analysis was conducted using Polo PC (LeOra Software 1987). The $aw_{50}$ and $aw_{90}$ values were estimated with their 95% fiducial limits. All values throughout the text are given with corresponding standard errors.

## Results

### *Effect of water activity levels on codling moth mortality*

The different volumes of water required to attain the desired level of water activity for each dish, are summarized in Table 1. All these water activity ($a_w$) levels caused some degree of larval mortality, except $a_w$ values < 0.92. Analysis of the results using a one-way ANOVA illustrated a significant positive effect of increasing water activity ($a_w$) on larvacidal activity ($F = 46.55$; $df = 11,108$; $P < 0.001$) (Figure 1). Very low levels of mortality (≤ 15%) were recorded at $a_w = 0.8 – 0.93$, whereafter mortality increased significantly from 15 ± 10.67 % at $a_w = 0.93$ to 95 ± 3..33% at $a_w = 0.95$ ($P < 0.001$). Mortality levels of ≥ 87% with no significant difference was observed at $a_w = 0.95 – 1.00$ ($P = 1.00$). The Probit analysis's regression equation for the combined data was $Y = 5.12 + 126.94$ [X], where Y = log ($a_w$). The $a_{w50}$ and $a_{w90}$ values and 95% fiducial limits were 0.94 (0.92-0.95) and 0.96 (0.95-1.00).

**Table 1.** The volume of water containing 300 infective juveniles or water only for control treatments, added to Petri dishes (3 cm diameter) lined with filter paper to attain corresponding water activity ($a_w$) – values.

| $a_w$ Value | Inoculum volume (µl) | $a_w$ Value | Inoculum volume (µl) |
|---|---|---|---|
| 0.8 | 6.0 | 0.95 | 16 |
| 0.9 | 8.0 | 0.96 | 18 |
| 0.91 | 8.5 | 0.97 | 20 |
| 0.92 | 9 | 0.98 | 24 |
| 0.93 | 10 | 0.99 | 26 |
| 0.94 | 12 | 1.00 | 28 |

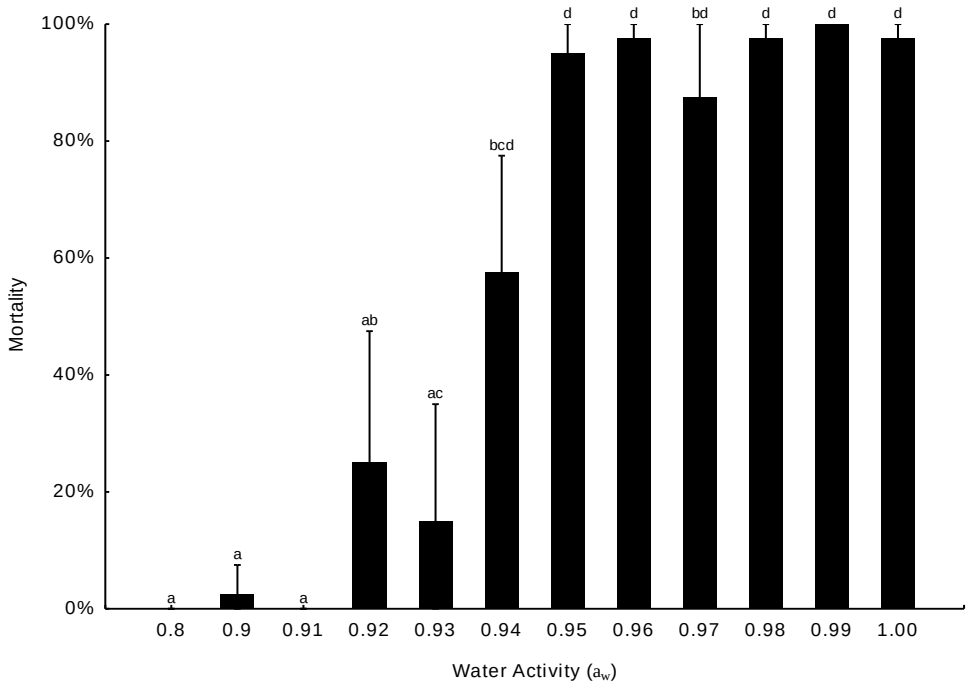


**Figure 1.** Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after exposure to 50 infective juveniles/larva of *Heterorhabditis zealandica* (SF41) at different levels of water activity ($a_w$). Different lettering above vertical bars indicates significant differences (one-way ANOVA; $F = 46.55$; $df = 11,108$; $P < 0.001$).

***Influence of different nematode concentrations and relative humidity on codling moth mortality***

Mortality was below 30% for all the levels of concentration tested (data not presented) at both 60% and 80% RH. Only the results obtained at 100% RH were therefore analyzed, using a one-way ANOVA ($F = 25.84$; $df = 5,54$; $P < 0.001$). A positive relationship between larvacidal activity and nematode concentration emanated from the results, with an incremental increase in mortality as nematode concentration increased (Figure 2). No mortality was observed when water only was applied. Relatively low levels of mortality (16 – 58%) were observed at nematode concentrations of 5 – 40 IJs/larva. Satisfactory levels of larvacidal activity (90 ± 6.61 %) were only recorded at 80 IJs/larva.

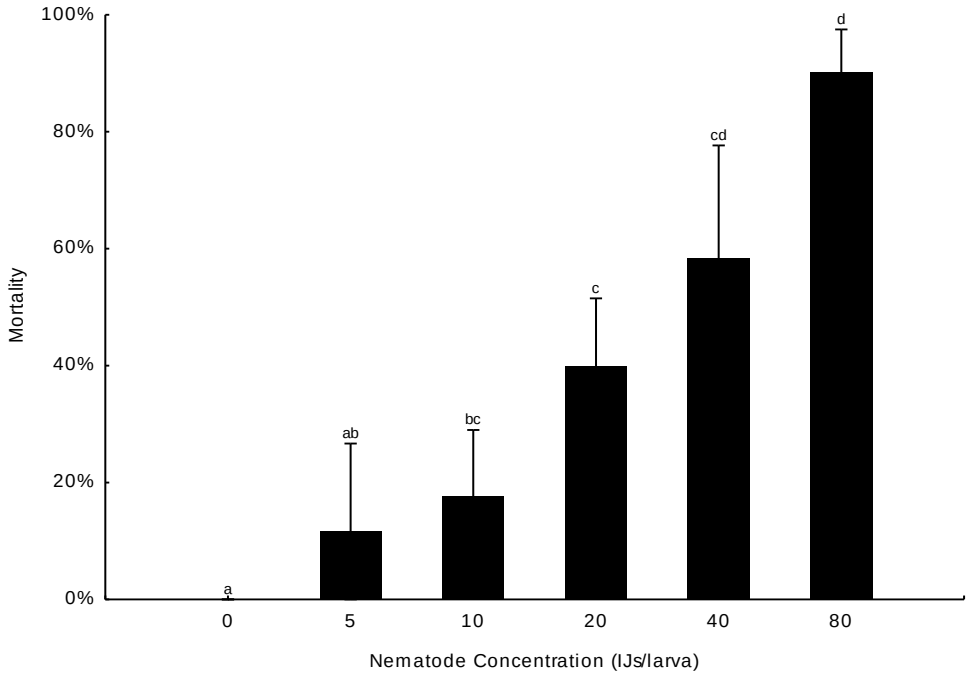


**Figure 2.** Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after exposure to different concentrations (infective juveniles/larva) of *Heterorhabditis zealandica* (SF41) at 100% humidity. Different lettering above vertical bars indicate significant differences (one-way ANOVA; $F = 25.84$; $df = 5,54$; $P < 0.001$).

***Influence of exposure time on codling moth mortality***

Larvacidal activity was recorded for each of the different exposure times (30 – 480 min) tested, with differences amongst treatments ($F = 10.21$; $df = 4,95$; $P < 0.001$). Significantly higher levels of mortality (ranging between 47 – 53%) were obtained after exposure to the nematodes for ≥ 240 min, as opposed to a shorter exposure time (30 -180 min) where mortality was below 25% (Figure 3) and no significant differences were observed ($P < 0.001$). Results analyzed with a one-way ANOVA therefore suggest that mortality significantly increases as exposure time lengthens to 240 min ($P = 0.002$), whereafter no additive effect was observed ($P = 1.00$).

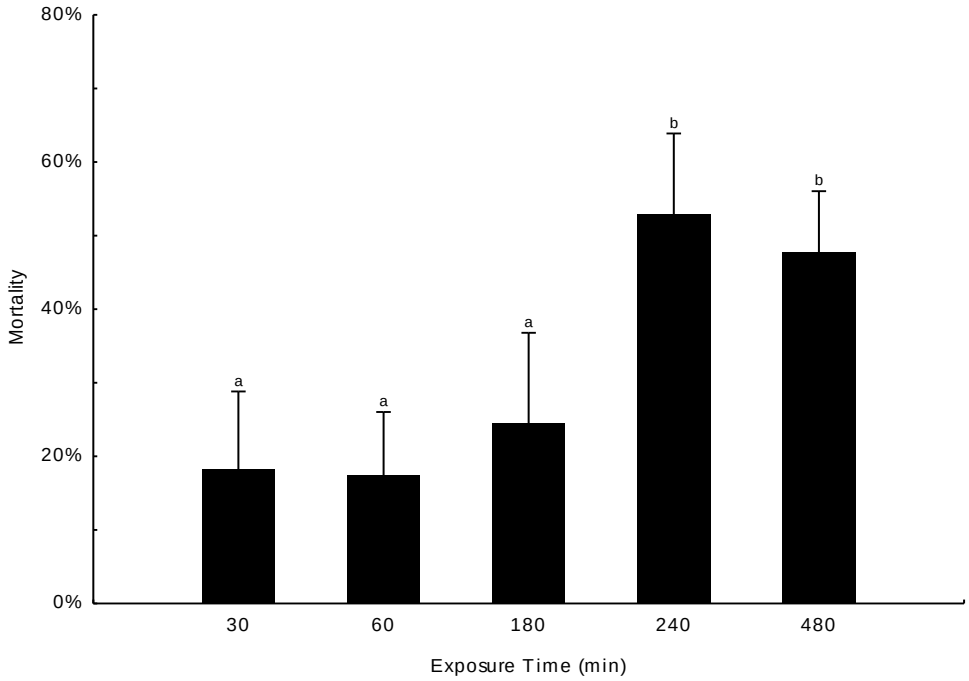


**Figure 3.** Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after exposure to 50 infective juveniles/larva of *Heterorhabditis zealandica* (SF41) for different lengths of time at 100% relative humidity. Different lettering above vertical bars indicate significant differences (one-way ANOVA; $F = 10.21$; $df = 4,95$; $P < 0.001$).

***Effect of a Zeba® formulation in tree trunk laboratory bioassay***

The two-way ANOVA (treatment x humidity) showed no interaction between the main effects ($F = 0.20$; $df = 1,116$; $P = 0.66$). Treatments behaved consistently over the two levels of humidity tested and main effects were therefore interpreted directly. This showed that the addition of Zeba® significantly increased the efficacy of the nematodes at both levels of humidity tested. At 60% RH,

mortality increased from 8 ± 4.15% to 23 ± 4.15% (P = 0.07) and at 80% RH from 17 ± 4.15% to 36 ± 4.15% (P = 0.01) (Figure 4). Using a one-way ANOVA, the data for both levels of humidity were pooled for a further analysis to illustrate that the use of Zeba® significantly increased the level of mortality obtained, from 12 ± 2.94% to 29 ± 2.94% (F = 16.32; df = 1,116; P < 0.001). Water activity measurements also reflected the additive effect of using Zeba®, as opposed to nematodes only, showing that water activity levels decreased for all treatments from $a_w$ = 1.00 directly post-treatment to $a_w$ = 0.93 (with Zeba®) and $a_w$ = 0.89 (without Zeba®) at 80% RH and $a_w$ = 0.70 (with Zeba®) and $a_w$ = 0.54 (without Zeba®) at 60% RH at the end of the trial period.

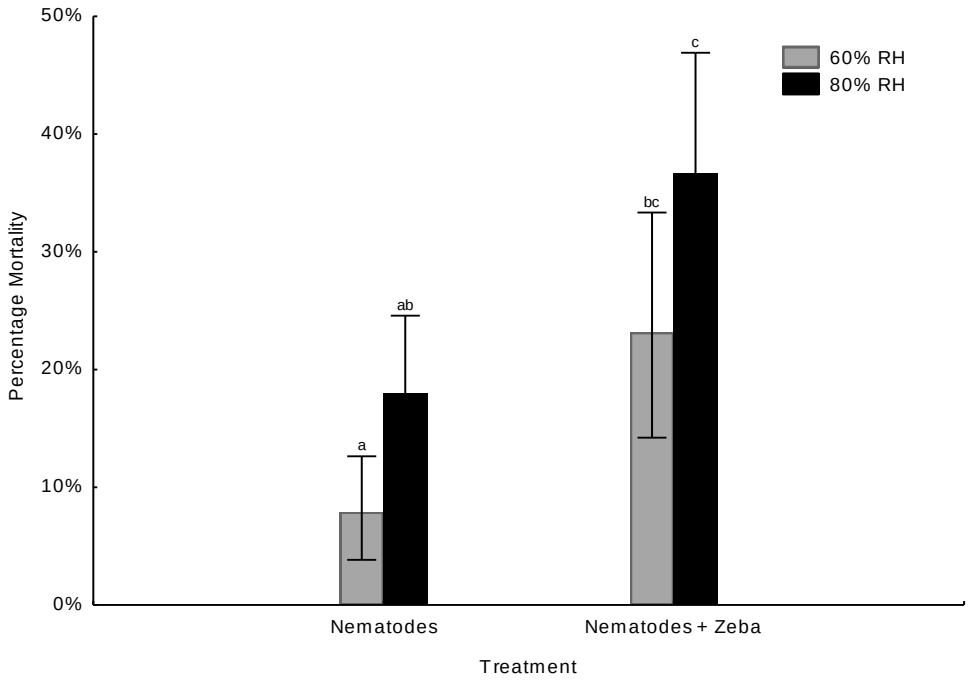


**Figure 4.** Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after exposure to *Heterorhabditis zealandica* (SF41) with and without Zeba® at 60 and 80% RH. Different lettering above vertical bars indicates significant differences (two-way ANOVA; F = 0.20; df = 1,116; P = 0.66).

***Field application***

Moderate temperatures (ranging between 12 and 30°C) were recorded throughout the trial period. The relative humidity in the orchard was low (≈ 35% RH) at the time of application and increased after

approximately 12 h due to light rain which was recorded inconsistently throughout the last 12 h of the trial period (Figure 4).

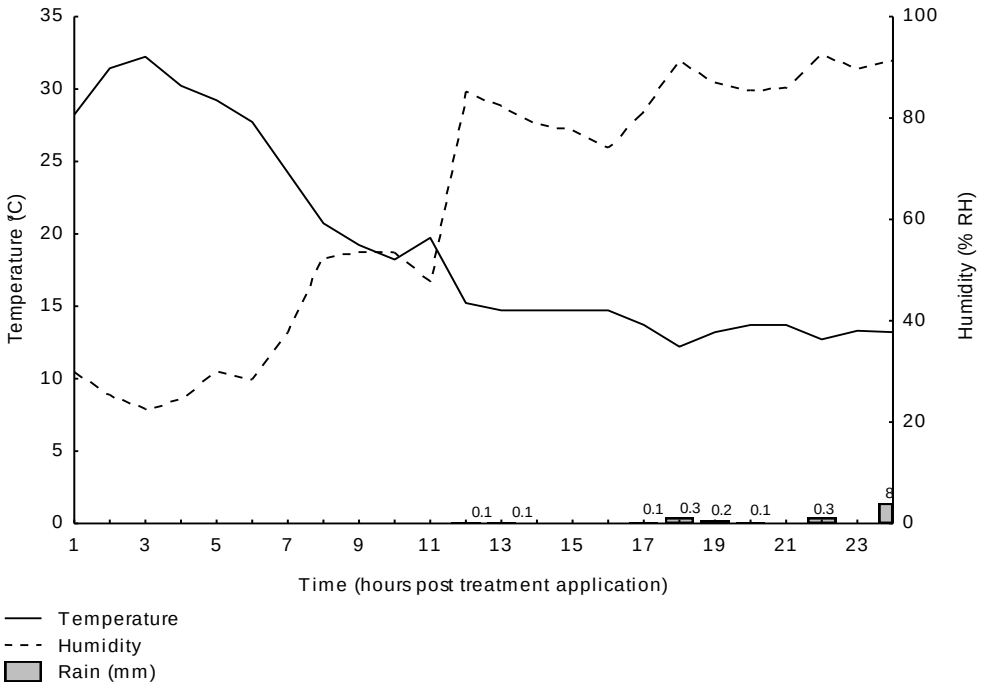


**Figure 5.** Climatic data recorded over a 24 h period during a field experiment.

Larval mortality recorded for the three trunks per tree were combined and analyzed, using a one-way ANOVA. Relatively equal levels of mortality due to nematodes were obtained for the three nematode treatments (T1: 45.64 ± 5.71%; T2: 46.13 ± 7.83%; T3: 45.38 ± 4.28), as opposed to the control treatment where the level of natural mortality was significantly lower (T4: 22.93 ± 1.31%) ($F = 4.55$; $df = 3,28$; $P < 0.01$) (Figure 6). Field results thus indicated that the addition of Zeba® (T2) and Zeba®+ Nu-Film-P® (T3) had no markable effect on larvacidal activity in the field.

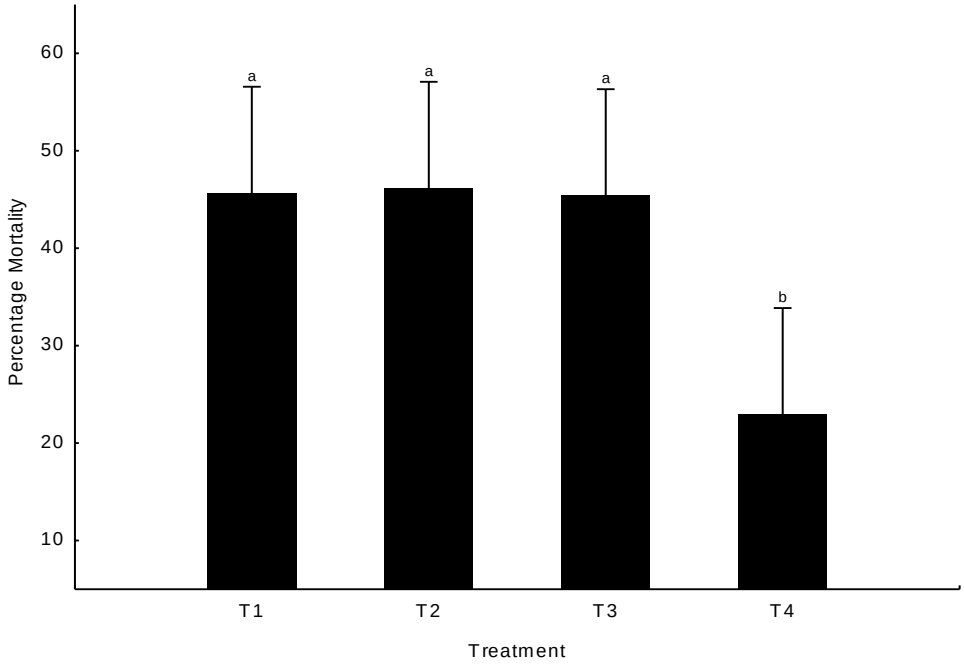


**Figure 6.** Mean percentage mortality (95% Confidence Interval) recorded for diapausing codling moth larvae after exposure to different formulations of *Heterorhabditis zealandica* (SF 41) during a field trial conducted in June 2011. Treatments were: (1) nematodes, (2) nematodes + Zeba®, (3) nematodes + Zeba® + Nu-Film-P® and (4) water as a control treatment. Different lettering above vertical bars indicate significant differences (factorial ANOVA; $F = 4.55$; $df = 3,28$; $P < 0.01$).

For all three nematode treatments tested, nematode survival rate declined as the trial progressed (Figure 7). The average survival rate for the formulated nematode treatments (Treatment 2: 45.01 ± 13.79 % and Treatment 3: 44.06 ± 11.01 %) was higher than for Treatment 1 (32.96 ± 13.79 %), where nematodes were applied with water only. The biggest decline in survival rate occurred during the first 3 h post-application, especially for Treatment 1.

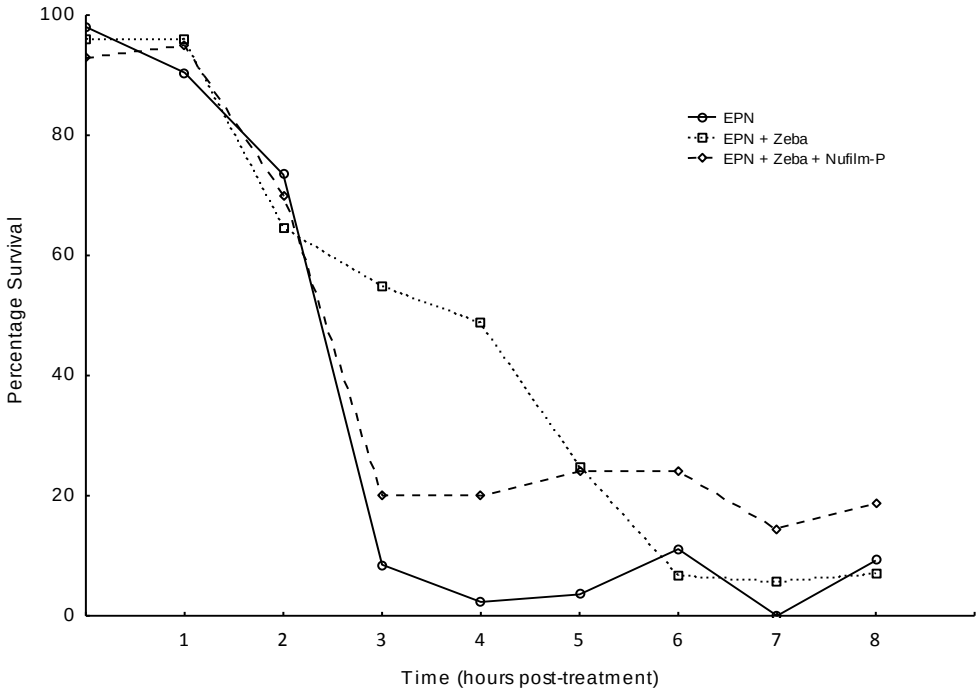


**Figure 7.** Percentage nematode survival recorded for *Heterorhabditis zealandica* (SF 41) on pieces of bark at different time intervals post-treatment.

For all three nematode treatments, nematode infectivity also declined as the trial progressed (Figure 8). Infectivity was only recorded for the first 3 h for Treatment 1, as opposed to Treatment 2 and 3, where infectivity was recorded for up to 4 and 5 h.

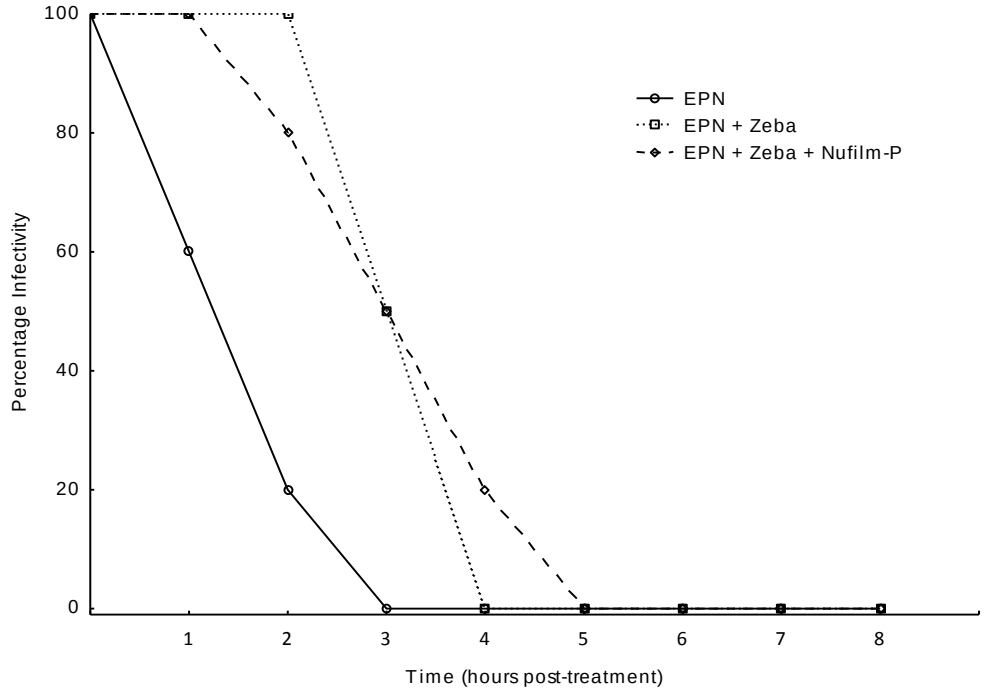


**Figure 8.** Percentage codling moth mortality recorded after exposure to *Heterorhabditis zealandica* (SF 41) for different time intervals during a field trial.

## Discussion

Literature indicates that *S. feltiae* and *S. carpocapsae* are the most promising nematode species for the control of codling moth and therefore the most widely used (Kaya *et al.* 1984; Lacey and Chauvin 1999; Vega *et al.* 2000; Unruh and Lacey 2001; Lacey and Unruh 2005; Lacey *et al.* 2006; Navaneethan *et al.* 2010). This is partially due to their commercial availability in most countries, but also because of their biological characteristics (Vega *et al*. 2000). In South Africa, however, neither of these two species has been isolated to date (Malan *et al.* 2006; De Waal 2008; Hatting *et al.* 2009) and current legislation prohibits the import of exotic species (Agricultural Pest Act 36 of 1947). As the study was conducted in South Africa, a locally-obtained isolate of *H. zealandica* was used. This isolate previously proved to be relatively effective for the control of codling moth (De Waal 2008; De Waal *et al.* 2010; De Waal *et al.* 2011). The current study contributed to developing a science-based solution to further increase the efficacy of this isolate, by overcoming the issue of nematode desiccation prior to larval infection, due to exposure to low levels of humidity, which, as was previously mentioned, is the biggest limiting factor for this type of application.

A clear distinction should be made between moisture-levels pertaining to the macro-environment (surrounding orchard humidity) and micro-environment (cryptic habitat where larvae, for example reside underneath loose pieces of bark on the tree), as conditions may differ considerably between these two environments (Navaneethan *et al.* 2010). Water activity ($a_w$-value) gives an indication of the available water in the cryptic habitats in the bark of trees, and thus the effectiveness of nematodes in this micro-environment, as nematodes need a water film for propulsion (Koppenhöfer 2007). Navaneethan *et al.* (2010) documented the first investigation of the influence of water activity on nematode efficacy, using *S. feltiae*. The current study elaborated on this concept, except that *H. zealandica* was used as a test isolate. Results indicated that *H. zealandica* was not necessarily dependent on a water film ($a_w = 1$) to infest codling moth larvae, as larvacidal activity was still recorded down to $a_w = 0.92$. To ensure a 90% mortality rate, results indicated that an $a_w$-value of at least 0.96 should be maintained on trees during an application. These results were similar to the findings for *S.*

*feltiae* in the aforementioned study, where larvacidal activity was recorded down to $a_w$ = 0.90 with $a_{w90}$ = 0.99 (Navaneethan *et al.* 2010). It has been noted that bark from living trees have an $a_w$-value of approximately 0.96 (Navaneethan *et al.* 2010), which, based on the results obtained, would support nematode activity and ensure infection to a relatively satisfactory level of control. However, as suggested by the positive relationship between increasing water activity and the subsequent level of control observed from the current study's results, even higher levels of control can be attained by the application of greater volumes of water. There is however the risk of over-wetting trees which would lead to run off and the subsequent loss of nematodes. Water is also a limited resource in most temperate regions, and therefore the benefit needs to be weighed against the cost for this recommendation.

Increasing the concentration of nematodes during an application previously showed that it enhances the eventual level of control (Navaneethan *et al.* 2010). Similar findings were obtained from the current study, where a positive relationship was observed between the number of nematodes and the eventual level of control, with the highest level of control was obtained when using 80 IJs/larva. The correlative relationship between the variable cost-factor involved in nematode production and the proposed increased amount of nematodes for enhanced efficacy, should however be taken into consideration before practical implementation.

As was previously mentioned, the ambient humidity in the macro-environment also contributes to the success of an application. In the concentration experiment it was illustrated that, where codling moth mortality was below 30% for all trials incubated at 60% and 80% RH, as opposed to 100% RH, where satisfactory levels of control (up to 90%) were obtained. These results were consistent with the findings of Lacey and Unruh (1998), where nematodes were only active at maximum levels of humidity (> 95% RH).

Optimal humidity levels need to prevail long enough to ensure the successful penetration of the insect by the nematodes (Lacey *et al.* 2006). The exposure-time experiment showed that exposure of the larvae to the nematodes for half an hour was already sufficient for larvacidal activity. Prolonging exposure time thereafter further increased mortality. This pattern was however only observed up to 4 h, whereafter no significant increase in mortality was recorded. This implies that the first 4 h post-application is the most crucial for ensuring successful larval infection. To ensure the efficacy of the

treatment, trees should therefore ideally be kept wet for this period. It is, however, important to remember that other environmental factors such as wind and temperature could also influence desiccation-rate, and it is therefore advisable to maintain high moisture levels in an orchard for as long as possible post-treatment. Previous exposure time trials conducted with *H. zealandica* showed that the degree of crypticness of larval location also influences the time required for the nematodes to reach and infect the codling moth larvae. When nematodes were, for example, applied directly onto perforated cardboard strips containing codling moth larvae only 5 h of high humidity were required to achieve 95% mortality, as opposed to a mulch treatment, where larvae were hidden approximately 5 cm below the substratum surface, and almost 3 days of optimal conditions were required for the nematodes to locate and infect 95% of the population (De Waal 2008; De Waal *et al.* 2011). Where pear tree trunks were used for insect containment in this study, host contact was relatively assured. Larvae were allowed to spin their cocoons in holes which were drilled 1 cm into the trunks and as the nematodes were applied directly onto the trunk surface, the only physical barrier through which the nematodes had to penetrate was the larval cocoons, which Navaneethan *et al.* (2010) showed not to be a limiting factor.

It has been noted that in several recent publications, improved formulation of nematodes increased the efficacy of aboveground applications for the control of certain orchard pests (Navaneethan *et al.* 2010; Lacey *et al.* 2010; Shapiro-Ilan *et al.* 2010). In this study, similar results were obtained in the laboratory formulation experiment, whereby the addition of Zeba® with the nematodes - as opposed to applying nematodes only - almost doubled the level of control obtained at 60% and 80% RH, and water activity levels on the bark also remained more consistent with the addition of Zeba®, which therefore favoured survival and subsequent efficacy of the nematodes.

During the field experiment, moderate temperatures (ranging between 12 and 30°C) during the field experiment would theoretically have ensured the survival of the nematodes (Koppenhöfer 2008). However, low levels of humidity (≈ 35% RH) during the first few hours after application would have been detrimental to the survival of the nematodes (Koppenhöfer 2008) and could partially explain the low levels of mortality obtained (≈ 55%) for all three nematode treatments. The non-significant difference in mortality obtained between the three nematode treatments containing either only nematodes, or nematodes in formulation: Zeba® or Zeba® + Nufilm-P®, would suggest that formulation evidentially had no probative effect on nematode efficacy. These results were confusing,

as the previous laboratory formulation experiment indicated an evidential increase in mortality with improved formulation. However, the nematode survival and codling moth infectivity experiments confirmed the aforementioned expected results as a more gradual decline in nematode mortality was observed on bark pieces when nematodes were applied in formulation, as opposed to applying nematodes only and a longer codling moth infection period was recorded for nematodes applied in formulation as opposed to non-formulated nematodes. Regarding formulation (Zeba® vs. Zeba® + Nu-Film-P®), results from both the nematode-survival and infectivity experiments, indicated that the use of both Zeba® and Nu-Film-P® yielded the best results and would in future possibly be recommended for the formulation of *H. zealandica* for the improved control of codling moth.

The study conclusively illustrated the required water activity levels in the micro-environment to ensure the survival and subsequent efficacy of *H. zealandica,* and it furthermore highlighted the importance of optimizing other contributing factors, such as exposure time and nematode concentration. Final results showed that the addition of the formulation enhanced the efficacy of a treatment by preventing rapid nematode desiccation due to low levels of humidity in the orchard environment. The use of the Zeba® formulation is therefore recommended for the improved control of codling moth in temperate regions, using *H. zealandica*.

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# CHAPTER 6

## Conclusion

The general aim of the study was to find science-based solutions to some of the complex environmental and biological factors impeding the use of local isolates of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) for the biological control of diapausing codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) in South Africa. This is the first report of its kind to comprehensively investigate this topic and to also illustrate how these ecological and biological factors can be manipulated to ensure the efficacy of treatments.

Wooden fruit bins are a known source of codling moth infestation in local orchards and their successful disinfestation would, therefore, greatly benefit the current control programme, which includes certain density-dependent control measures. The first objective of the study was, therefore, to identify some of the baseline requirements in a small-scale experiment for the successful disinfestation of wooden fruit bins of diapausing codling moth larvae, using the local SF41 isolate of *Heterorhabditis zealandica* Poinar 1990, which has previously not been evaluated for bin-treatments. Prior to full-scale treatments, preliminary bioassays were first undertaken in the current study, which would in future, form the basis of the operational protocol to be implemented on a commercial scale. Nematode application rate for bin treatments proved to be directly proportional to the eventual level of codling moth mortality obtained, and an optimal concentration for maximum mortality was established to be 100 IJs/ml. A pre-wet treatment for 1 min and subsequent incubation for approximately three days at temperatures > 15°C and humidity > 90% RH, favoured nematode survival and proved to be imperative to the success of a bin treatment. Tarping bins post-treatment and the addition of the adjuvant Reverseal® further enhanced the eventual level of control. Results emanating from Chapter 2 conclusively illustrated that *H. zealandica* could be used for the successful disinfestation of fruit bins. This is the first study that clearly indicates that the success of a treatment is highly dependent on the handling conditions, as documented throughout Chapter 2. Future research should be aimed at developing the technology required for full-scale treatments of bins, considering practical and logistical aspects associated with commercial bin-handling.

Mulches have also been noted to be a source of codling moth infestation, and as previously mentioned, the disinfestation of these codling moth sources, would benefit current density-dependant control programmes. As mulching is becoming increasing popular in local orchards, the potential risk of codling moth infestation also increases and the successful use of nematodes to control diapausing codling moth larvae in mulch layers would, therefore, be beneficial, as no current biological mulch-disinfestation method exists. The objective of Chapter 3 was, subsequently, to investigate the potential use of the SF41 isolate of *H. zealandica,* together with different mulches to control diapausing codling moth larvae. Cylindrical mesh cages were identified as a suitable insect containment device, facilitating the easy removal of codling moth larvae from mulches post-treatment during experimentation. Mulch type also proved to impact the efficacy treatments evidentially. The best results were obtained using pine wood shavings as opposed to pine wood chips, wheat straw, blackwood or apple wood chips. Local growers, however, favour wheat straw and apple wood chips for mulching, and these mulches were, therefore, selected for further experimentation. It was also proven, that should nematodes be washed out of the mulch layer due to heavy rains, as can be expected at the proposed time of application, nematodes would have the ability to move out of soil back into the mulch to infect codling moth larvae residing at certain heights. Abiotic factors pertaining to conditions in the mulch layer, such as temperature and humidity, also proved to be crucial to the survival and subsequent efficacy of nematodes in test mulches. The study conclusively illustrated the important baseline requirements fundamental to an application and that, if these requirements were to be met, *H. zealandica* would effectively control diapausing codling moth larvae in mulches. This is the first study to investigate *H. zealandica*'s use in this regard, and the results are of great value to the local fruit industry for the future biological disinfestation of mulches. Based on the promising results obtained from the study, future research should be directed at evaluating the use of nematodes to target other pest insects also occurring in mulch layers. The use of mulches in conjunction with nematodes for the control of codling moth would also most probably only be applicable to apple orchards, as apple trees generally have a smoother bark than do pear trees, thereby offering less suitable cryptic sites for codling moth to overwinter in. Consequently, codling moth larvae would be more prone to overwinter in mulch layers at the base of trees, as opposed to overwintering on the tree itself, as would be the case with pear trees.

The biological and ecological characteristics pertaining to the nematode isolate and the environmental conditions associated with codling moth need to be matched in order for a nematode isolate to be

effective. The third objective of the study was, therefore, to evaluate the biocontrol potential of South African isolates of: *Steinernema citrae* (141-C) Stokwe, Malan, Nguyen, Knoetze and Tiedt 2011; *Steinernema khoisanae* (J69 and SF87) Nguyen, Malan and Gozel 2006; *Steinernema yirgalemense* (157-C) Nguyen, Tesfamarian, Gozel, Gaugler and Adams 2005; an undescribed *Steinernema* sp. (J194) and *Heterorhabditis zealandica* (SF41), against diapausing codling moth larvae. Most of these isolates have not previously been tested against codling moth. Codling moth proved to be highly susceptible to all tested isolates (mortality 78 – 100%). Low temperatures (≈ 15°C) and low moisture levels ($a_w \leq 0.92$), as can be expected in the field during application, negatively affected all isolates. Field experiments were conducted with *H. zealandica, S. khoisanae* and *Steinernema* sp., which showed all three isolates tested to be moderately effective (mortality 52 - 70%) against codling moth under field conditions. Different insect containment methods (wooden planks vs. pear tree logs vs. mesh cages) used during field experimentation were also shown to influence larvacidal activity, and predictive equations were subsequently derived. Future trials can, therefore, be conducted with user-friendly insect containment devices (wooden planks and mesh cages), whilst being able to predict what the expected level of control for the natural situation (pear tree logs) would have been. The general objective of Chapter 4 was successfully met; however, a single effective nematode isolate for the control of codling moth, was not identified, and further research should, therefore, be aimed at obtaining an isolate that is effective at low temperatures and low levels of humidity as can be expected in the field during application.

In all chapters, low moisture levels were noted to be the most limiting factor to the survival of the nematodes during an application aimed at codling moth. The final objective of the study was therefore to determine whether nematode formulation with a superabsorbent polymer, Zeba®, could be used to improve the performance of the SF41 isolate of *H. zealandica* for the control of codling moth. The study illustrated the required moisture level ($a_w \geq 0.92$) to ensure the survival of *H. zealandica,* and, furthermore, highlighted the importance of optimizing other contributing factors, such as exposure time (≥ 4 h) and nematode concentration (≥ 80 IJs/larva). Results showed that the addition of Zeba® enhanced the efficacy of a treatment, by preventing rapid nematode desiccation resulting from low levels of humidity in the orchard environment. These results can, therefore, be used as part of the future recommendation for the enhanced efficacy of a nematode treatment aimed at codling moth. It would, however, be worthwhile to investigate the economic-viability of the costs associated with this type of recommendation and to determine the benefit-cost relations, before practical implementation.

In general, all objectives were successfully met, thereby illustrating most of the biological and ecological requirements fundamental to the successful use of entomopathogenic nematodes for the control of codling moth in local pome fruit orchards. Results emanating from the proposed future research topics should be combined with the current study's results and subsequent recommendations, to ensure that the use of entomopathogenic nematodes for the control of codling moth is affordable, effective and practical.

The commercial application of this technology would lead to the reduced use of pesticides, thereby reducing the risks associated with pesticide-use, such as concerns over human safety, environmental impact and the general sustainability of synthetic pesticides in agro-ecosystems.